THE JOHNS HOPKINS UNIVERSITY
SCHOOL OF HYGIENE AND PUBLIC HEALTH
310-312 W. MONUMENT STREET
BALTIMORE, MD

DR. LISK'S THESIS

PUBLISHED

JOURNAL OF BACTERIOLOGY

VOL. IX, 1924, p. 1-12

A STUDY OF THE DECOMPOSITION PRODUCTS OF SPORE-BEARING BACTERIA IN HEATED MILK.

By

HENRIETTA LISK

Thesis

Submitted in Partial Fulfilment of the Requirements

for the

Degree of Doctor of Science

in Hygiene

in

The School of Hygiene and Public Health

Johns Hopkins University

1923.

TABLE OF CONTENTS

INTRODUCTION

Years ago it was pointed out by Flügge(18) 1894 that milk contains resistant spores of many species of bacteria which are able to bring about putrefactive changes under suitable conditions. Some of these spores were extremely resistant to the action of heat, withstanding boiling for long periods, eight hours or more. Flügge suggested that such spores taken into the alimentary canal of children would withstand the destructive action of the gastric juice, pass through the small intestine, multiply in the large intestine and there produce putrefactive products which might act as an irritant to the intestinal mucosa and thus play a role in the production of summer diarrhoea. It was subsequently shown by Ford and Pryor (26) 1904 that in decomposing heated milk the aerobic spore-bearing bacteria described by Flügge were always present and these species were carefully described by Lawrence and Ford (41) 1916. It was also shown by Ford and Williams (27) 1919 that heated milk kept in a warm place (37^{o}) decomposes with the elaboration of products poisonous to animals and some of these products were traced to the growth of Bacillus Welchii (Bacillus aerogenes capsulatus). In a subsequent discussion of the products of putrefaction from several anaerobes it was pointed out by Wolf and Harris (79) 1916-17 that no adequate study of the products of putrefaction from aerobic spore-bearing bacteria had ever been made.

In view of the universal use of cow's milk as a food for man, especially for children, and in further consideration of the constant

presence of such organisms in milk as shown by Flügge, and Ford and Pryor this investigation was undertaken to determine the nature and extent of the decomposition of milk from the predominant aerobic spore-bearing bacteria found in cow's milk in Baltimore. In order to make clear the results of the investigation actually undertaken and carried out, a thorough review of the older work dealing with putrefaction and the methods employed by earlier investigators was undertaken and this review has also been included in the hope of clearing up some of the prevailing confusion as to the meaning of the various terms employed.

LITERATURE.

(A) Putrefaction.

According to Descour (15) Pasteur first demonstrated anaerobic life in his studies of butyric fermentation (1861). In a meeting of the Academy of Medicine, March 9,1875, Pasteur, in discussing the ways in which, after death, organic matter returns to the atmosphere or soil, described the transformation of a mass of grapes placed in a vat. He noted the change into wine, then into vinegar, and the final appearance of the "foul odor which tells of putrefaction." Down at the bottom layers of the mixture the anaerobic vibrions of putrefaction decompose a large part of the macerated solids with the production of sulphuretted hydrogen, ammonia and other evil-smelling gases.

Tissier and Martelly (62), in a review of the literature on putrefaction, state that Pasteur first demonstrated that putrefaction was the work of anaerobic bacteria, but they believe that, "according to the studies of Hauser, Kühne, Foa and Bonome, Tito-Carbone, Lannelongue and Achard on Bacillus Proteus, we must admit that the aerobes also play a part in putrefaction". Malvoz, in 1890, attributed putrefaction to Bacillus coli. Little by little the anaerobes were forgotten and Mace in describing the phases of putrefaction, passed over them in silence. Veillon and Zuber, Halle, Rist, Guillemot and Cottet, in a series of reliable investigations demonstrated that anaerobes existed constantly and often even uniquely in all pus having a putrid odor.

Rienstock (4) 1899-1901 showed that putrefaction was caused by anaerobes but Tissier and Martelly concluded that putrefaction is the work of both aerobes and of anaerobes. They describe the putrefaction of beef in two phases. In the first phase, both the sugar and protein were attacked by mixed ferments, proteolytic and saccharolytic. In the second phase, the protein and its products were attacked by ferments which were proteolytic or peptolytic. (Peptolytic is a term applied to ferments which attack protein only after its reduction to peptone.)

Tissier and Gasching (69) 1903, investigated the fermentation of milk and concluded that the progress of the fermentation was remarkably similar to that of the fermentation of beef; the chemical and physical composition of the media causing some differentiation. They believed that all the species causing habitual putrefaction in milk, that is, complete destruction of all its elements, are contained in the milk when it leaves the dairy. In the samples examined bacteria and fungi were found constantly. The bacteria were divided into two classes.

(1) Mixed ferments (a) Proteolytic. Ex. Staphylocoque.
(b) Mixed peptolytic ferments. Ex. Entérocoque, Bacillus coli, Bacillus acidi lactici, and Bacillus lacto-propyl-butyricus.

(2) Simple ferments (a) Simple proteolytic ferments.
Ex. Bacillus mesentericus, Bacillus subtilis, Bacillus putrificus, and Proteus vulgaris.
(b) Simple peptolytic ferments.
Ex. Proteus Zenkeri, Bacillus fecalis alkaligenes.

The fungi were Oedium lactis, and Rhizopus nigricans.

The progress of putrefaction in milk was found to be always the same. The simple ferments peptonized and destroyed the casein, but, de-

veloping simultaneously with the mixed ferments, their action was soon arrested by the acidity of the medium produced by the mixed ferments. Oedium lactis and Rhizopus nigricans now multiplied and destroyed the acids and the remaining lactose of the milk, and the simple ferments then completed the destruction of the casein and its derivatives.

Rettger (53), 1903 made a study of the chemical products of Bacillus coli communis and Bacillus lactis aerogenes in a culture medium consisting of coagulated egg white and lean beef. He concluded that not only the obligatory anaerobes, but the facultative aerobes, possess the power of inaugurating putrefactive changes in proteids. In 1906 Rettger (54) stated that in later work with these organisms, he found that anaerobes were present as contaminations in his cultures, and that, when sterile egg meat was inoculated with pure cultures of Bacillus coli, and Bacillus lactis aerogenes, there was no decomposition. He also tested Bacillus fecalis alkaligenes, Proteus vulgaris, Bacillus pyocyaneous, Bacillus fluorescens, Bacillus cloacae, Bacillus prodigiosus, Staphylococcus pyogenes aureus and albus, Micrococcus cereus albus and Streptococcus pyogenes, with negative results. He, therefore, came to the conclusion that none of the known facultative anaerobes have the power to produce "genuine putrefaction."

Putrefaction as defined by Rettger is a bacterial decomposition of albuminous matter, which is accompanied by the formation or elimination of foul-smelling substance. In 1908, Rettger (55) states that "no one can deny that much of the decomposition of albuminous matter in nature is carried out by the obligate aerobes, but such transformation is one of ordinary dissolution or digestion." "Decomposition of albumin by the aerobes is never accompanied by the foul odors which are character-

istic of putrefaction."

Metchnikoff (47) 1908 concluded that aerobes produced putrefaction, but that the principal role was assumed by anaerobes.

According to Rettger and Newell (56) 1912-13 there is no word in the English language which exactly conveys the meaning of the word "faulniss", but the term putrefaction is of practically the same significance. It is derived from the Latin "putrere" meaning to be "rotten", and hence may be defined literally as a process of "rotting", or "offensive decay".

"Putrefaction", they say, "may be defined as a particular process of protein decomposition, which is brought about through the agency of bacteria with the evolution of foul smelling products, which are characteristic of ordinary cadaveric decomposition". Meroaptan formation they consider of particular significance.

Rettger and Newell further state that many of the common aerobes and facultative anaerobes have definite proteolytic action when in contact with free oxygen. In a study of the proteus group, these authors found that the twenty-six strains studied showed no ability to initiate and bring about a visible change in the character of the medium when grown under anaerobic conditions. When grown under aerobic conditions, the results were also negative as far as "actual putrefaction" was concerned. Proteus vulgaris was found to attack, and to a certain extent, decompose the egg meat proteins and blood fibrins. The action, however, was slow and dependent upon an ample supply of free oxygen. Such a decomposition was accompanied by the formation of such products as indole, amines, amino acids, and hydrogen sulphide. Rettger and

Newell did not consider these products as indicative of "real putrefaction", and further state that the odor given off was not that characteristic of putrefactive changes. These authors believe that Proteus vulgaris, together with other organisms which are essentially aerobic, "exerts an early disintegrative action during the process of which free oxygen is removed from the medium, whereby the putrefying anaerobes are enabled to carry on the process of real putrefaction." They concluded that putrefaction is the work of certain obligate anaerobes, and, if such a function is assumed by any other organism, it is rare and of little significance.

Kendall and Farmer (36) 1912-13 define putrefaction as "the action of micro-organisms upon nitrogenous substances", while fermentation is defined as "the action of micro-organisms upon carbohydrate."

Kendall (35) 1916 also states that the terms fermentation and putrefaction have been confused and even used synonomously in bacteriological, chemical and legal nomenclature, but that they represent two distinct types of bacterial activity. Fisher, according to Kendall, has defined fermentation in the broad sense in which it should be used as "the bio-chemical decomposition of nitrogen free compounds, chiefly carbohydrates by the action of micro-organisms", while "putrefaction is the bio-chemical decomposition of nitrogenous organic compounds by the action of micro-organisms".

As has been shown in the above review of the literature dealing with putrefaction the definition of the term as accepted by the various authors differs widely. Some writers have considered a breaking down

of proteins to amino acids as putrefaction, while others have claimed that not only must the hydrolysis proceed beyond the formation of amino acids, but these end products must have a foul odor, such as is given by mercaptan.

In describing the action of aerobic spore-bearing bacteria upon milk, the word decomposition has been used to signify the breaking down of proteins and carbohydrates with the formation of ammonia, amino acids, indole, hydrogen sulphide, mercaptan, or other products, such as aldehydes, ketones and acids.

B. Quantitative Determination of the Products of the Nitrogenous Metabolism of Bacteria.

A review of bacteriological literature has shown that investigations of the nitrogenous metabolism of bacteria have not, until the present time, included a quantitative determination of the products of the decomposition of milk by aerobic spore-bearing bacteria. Indeed but few workers have included in their researches, a quantitative determination of the products of the metabolism of aerobic spore-bearing bacteria in any medium.

Kendall and Farmer(37,38) 1912, and Kendall, Farmer, Bagg and Day(39) 1912, carried out a series of investigations of the changes in total nitrogen and ammonia content of sugar free broth and dextrose broth cultures of aerobic bacteria. Bacillus mesentericus, an aerobic spore bearer, was included in these investigations. Kendall and his co-workers concluded that ammonia formation was an index for determining "the rate, and to a degree, the extent of protein catabolism by these bacteria."

Robinson and Tartar (61) 1917, working with two aerobic spore bearing bacteria, Bacillus subtilis and Bacillus mycoides, and with Bacillus vulgaris, determined quantitatively the changes in the total nitrogen, amide nitrogen, humin nitrogen, diamino-acid nitrogen, monoamino-acid nitrogen, and the ammonia content of a medium in which blood fibrin, egg albumin, or peptone furnished the protein. To these proteins were added a synthetic solution, containing mannite, secondary potassium

phosphate, calcium chloride, magnesium sulphate and ferric chloride. A second series of experiments were made using Bacillus subtilis as the organism and casein and gliadin as the proteins. Robinson and Tartar concluded that all the nitrogen forms were changed more or less by the action of bacteria and that the end product ammonia was formed. They further concluded that the mono-amino acid nitrogen, and the diamino-acid nitrogen of the protein were the chief sources of the ammonia formed by bacterial action. They also stated that "the similarity of chemical changes between the action of acid hydrolysis and the action of bacteria indicates that the latter is largely hydrolysis to the point of formation of amino acids."

Wolf and Harris (79) 1917 included ammonia and amino nitrogen formation in their investigations of the bio chemistry of Bacillus Welchii and Bacillus sporogenes. These authors found a constant increase in ammonia in the following media: milk, peptone water, alkaline casein, alkaline egg, and erepton solution. The clear liquid of a cooked meat medium was also used in the study of Bacillus sporogenes. Bacillus Welchii decreased the total amino nitrogen content in erepton solution when incubated for seventy-three and ninety-five hours. Bacillus sporogenes caused a decrease in amino nitrogen when cultures in peptone water were grown for twenty-four and for one hundred and fourteen hours. A decrease in amino nitrogen was also shown in erepton solution when cultures were incubated for ninety-three hours, and in cooked meat liquid when incubated for ninety-one hours. Both Bacillus Welchii and Bacillus sporogenes produced an increase in the amino nitrogen content of milk, alkaline casein and alkaline egg medium. Wolf and Harris (80) 1918-19, in working with Bacillus histolyticus found that this organism increased the ammonia and

the amino-nitrogen content of milk, peptone water, alkaline egg and cooked meat. Wolf (77) 1918-19 (b) found that the ammonia production of Vibrion septique served to differentiate it from Bacillus Welchii, for, while the ammonia production of Bacillus Welchii was slight, the rise in the concentration of ammonia in cultures of Vibrion septique was slow but definite, the final amount being about three times as great as that found in the sterile medium. Vibrion septique produced an increase in the ammonia and amino nitrogen content of tryptic broth, milk, glucose, peptone and cooked meat. Wolf (78) 1918-19 (c) found that Bacillus sporogenes, and Bacillus Welchii increased the total ammonia, and amino nitrogen content of cooked meat.

Harris (31) 1919-20 confirmed the observations of Wolf and Harris that Bacillus sporogenes decreased the total amount of amino nitrogen in peptone water.

Waksman (74) 1918 inoculated a synthetic medium, composed of secondary potassium phosphate, potassium chloride, magnesium sulphate, ferrous sulphate, saccharose and water, to which either peptone or casein were added, with various molds, and with Bacillus mycoides, an aerobic spore-bearing bacillus. All cultures showed an initial increase in amino nitrogen. In the case of Aspergillus niger this disappeared at the end of four days, but the remaining molds, and Bacillus mycoides, showed a continual increase in amino nitrogen to the end of the examinations, which were made on the first, fourth, seventh, and fourteenth days. Waksman concluded that all of these organisms formed a large amount of amino nitrogen and a smaller amount of ammonia. He states that "a study of ammonification is of doubtful importance in revealing the proteolytic activities of micro organisms, for it has not yet been proven that

ammonia is an end product of protein metabolism."

Falk, Baumann and McGuire (18) 1919 studied the changes taking place in the total nitrogen, the non-protein nitrogen, the ammonia nitrogen, the total creatinine nitrogen (creatine plus creatinine), and the purin nitrogen during the spoilage of meat. The organisms employed were Bacillus proteus, Bacillus coli communis, Bacillus paratyphosus β, Bacillus enteritidis, Bacillus subtilis and Streptococcus brevis. "Owing to the difficulty if not the impossibility of working with sterile meat" these workers used meat extracts as culture medium. Their results were as follows: The creatine-creatinine values changed only slightly with Bacillus proteus, and Bacillus paratyphosus β, but decreased markedly with Bacillus coli communis, Bacillus enteritidis, Bacillus subtilis, and Streptococcus brevis. The purine values decreased rapidly with Bacillus coli communis, and Bacillus enteritidis. With bacillus proteus and Bacillus paratyphosus β, the purine nitrogen showed a more or less irregular variation, which was accidental, followed by a slight decrease. Bacillus subtilis and Streptococcus brevis gave a distinct final increase in the purine indicating a synthesis. The ammonia values increased in every case.

In 1920, Waksman (75) in a study of the nitrogen metabolism of Actinomycetes, concluded that the production of amino acids is not a waste process resulting from growth, but that it represents a definite step in the metabolism of the organisms.

Foster (29) 1921 observed that a decrease in amino nitrogen was associated with an increase in ammonia in cultures of Streptococcus hemolyticus.

Raistrick and Clark (50) 1921, found that the total amino nitrogen content of bacterial cultures, in which tryptophane and tyrosine were the only nitrogen sources, decreased both in the absence and presence of glycerol.

Kendall, Day and Walker (41) 1922, investigated the chemical changes produced in plain broth, gelatin, milk and media containing mannitol, glycerol, lactose, saccharose and starch, by certain anaerobic bacteria. In cultures of Bacillus Welchii in plain broth, gelatin, glucose, glycerol, and starch, there was a constant and usually progressive increase in ammonia from the first to the thirteenth day. In mannitol, lactose, saccharose and milk, there was in some instances an increase of ammonia after one day's growth, while in other cases a decrease was noted. In one culture grown in saccharose medium this decrease was noted in the first to the sixth day. The amino nitrogen content of all media with the exception of milk showed a constant, though not always progressive increase from the first to the thirteenth day. In milk there was observed a decrease of amino nitrogen on the first day, and this decrease was found to exist for variable lengths of time, ranging from the first day only to the twentieth day of growth. In one instance, however, there was recorded an increase in the amino nitrogen content of milk on the first day and the thirteenth day of growth.

Vibrion septique was observed to produce a constant increase in ammonia from the first to the twentieth day. Amino nitrogen, however, fluctuated from increase to decrease in every medium with the exception of plain broth. The periods for which this decrease was recorded varied from the first to the ninth day of growth in the starch medium.

Bacillus fallax was found to vary in its production of ammonia and amino nitrogen. In some instances an increase was recorded throughout the examination, while, in other instances, the same medium showed a decrease varying from the first to the twenty-first day.

Bacillus sporogenes showed with one exception a constant and progressive increase in ammonia from the first to the twentieth day. Amino nitrogen values fluctuated from increase to decrease in every medium used with the exception of milk. With the remaining media used there was in every case to be found a constant increase from the first to the twentieth day to be contrasted with a decrease in amino nitrogen in the same medium. In glucose medium this decrease existed for the first day's growth only, but in mannitol, lactose, saccharose and starch the decrease was existent on the thirteenth day. In plain broth one culture produced a progressive increase in amino nitrogen from the first to the twentieth day, while another culture showed an increase on the first day followed by a fluctuating decrease on the third, sixth, thirteenth and twentieth day. In the majority of cultures grown, however, Bacillus sporogenes showed an increase in amino nitrogen on the sixth day, and thereafter.

Bacillus histolyticus produced a constant and usually progressive increase in ammonia content from the first to the twentieth day. Amino nitrogen was found to fluctuate on the first day from decrease to increase, but, in the majority of instances, a definite increase was recorded on the third day and on succeeding examinations.

Bacillus putrificus increased the ammonia of every medium used with the exception of glucose, which showed a decrease on the first and third day. Plain broth, gelatin, and saccharose media showed an initial decrease lasting until the third day and followed by an increase on the

sixth, tenth and fourteenth days. Glucose, lactose and milk showed variable results.

Bushnell (7) 1922 made a quantitative study of the bio chemical changes produced in various media by a type of anaerobic bacteria isolated from spoiled canned asparagus. Bushnell found that it was impossible to secure duplicates which checked exactly and states that "variation is inherent in all bacteriological work". He concludes, however, that quantitative results give a clear picture of the bio chemical behaviour of different types of organisms. He further expresses the hope that chemists will interest themselves in the improvement and simplification of chemical methods necessary to determine quantitatively and biochemically changes due to the growth of micro organisms in various culture media.

De Bord (13) 1923 investigated the nitrogenous metabolism of Bacillus coli, Bacillus pyocyaneus, Bacillus subtilis, Bacillus botulinus and Bacillus sporogenes. He thus included non-spore-bearing and spore-bearing aerobic bacteria and anaerobic spore bearers in his researches. Bacillus coli was chosen for "its active fermentive power." "Bacillus pyocyaneus was taken as a type which exhibits weak fermentive power and produces large quantities of ammonia." "Bacillus subtilis, Bacillus botulinus and Bacillus sporogenes were selected from the family Bacillaceae, which actively decomposes protein." A synthetic medium, containing peptone, mono-potassium and disodium phosphates, and glucose when a carbohydrate was desired, was used throughout these investigations.

Bacillus coli produced a constant but not progressive increase in ammonia content from the first to the tenth day of growth. In media which differed only in the amount of phosphorous, Bacillus coli produced in one instance a constant though fluctuating increase in ammonia nitrogen content, while in another instance amino nitrogen was decreased until the tenth day when the final content was slightly higher than the control.

In medium which was identical in composition, Bacillus pyocyaneus

showed an initial decrease in ammonia on the first day followed by a progressive increase, but in a second analysis recorded a progressive increase from the first to the tenth day. Amino nitrogen increased in all instances recorded, though not progressively.

Bacillus subtilis produced a constant increase in ammonia. The amount of ammonia formed in ten days in two comparable analyses, however, differed by 46.3 mgms per 100 cc of the medium. Amino nitrogen showed a constant but fluctuating increase from the first to the tenth day. The amounts formed in the two series of analyses differed on the tenth day by 24.3 mgms per 100 cc of the medium.

Bacillus botulinus showed an increase in ammonia from the first to the tenth day. Amino nitrogen formation fluctuated - a decrease being shown on the fourth and tenth day in one analysis, and on the first and second day in another analysis. De Bord states that the drop on the second and fourth day corresponded to the times when the bacteria showed a definite decrease in numbers. Spores could not be demonstrated in stained smear preparations, but a large number of shadow forms were noted.

Bacillus sporogenes produced a constant but not progressive increase in ammonia from the first to the tenth day. Amino nitrogen increased but not progressively. In one instance a decrease was noted on the fourth day of growth.

De Bord concluded that the rate of production of amino nitrogen or ammonia nitrogen indicates different types of metabolism of bacteria.

As this review of the applications of quantitative methods to the determination of ammonia and amino nitrogen has shown, not only do the results of the various investigators differ rather widely but considerable variation is shown in results obtained by the same worker.

C. Discussion of previous methods of chemical analysis

Ammonia Determination

Boussingault (6) 1850 published a paper in which he described a method for determining ammonia in solutions of its salts and in urine by distilling to dryness in a vacuum with milk of lime at 40° to 50°C. Sodium carbonate and bicarbonate were also used in the place of lime. The distillation was continued for about one hour. The ammonia was absorbed by standard acid during the distillation and then titrated.

Schlösing(62) 1851 described a method for the determination of ammonia in tobacco. Neubauer(46) in 1855 published a paper in which he described the application of Schlössing's method to the determination of ammonia in urine. The method consisted of adding magnesia or milk of lime to urine, and placing the dish containing this mixture in a bell jar together with another dish containing acid. At the end of 48 hours, Schlösing and Neubauer claimed that all the ammonia had been liberated and driven off from the liquid by the alkali and absorbed by the acid.

Würster(82) 1887 proposed a method for determining ammonia by distillation in a vacuum at low temperature. He distilled varying amounts of urine with baryta water, magnesia or lime, and claimed that at 15 mm pressure and 50°C, the operation was completed in 5, 10 or 15 minutes when 5, 10 or 20 cc. of urine were used.

Folin (20) 1901 described a method based upon the distillation of urine with magnesia in two periods of the same length. The differ-

ence between the ammonia received during the second period (which was formed by the decomposition of the urea) and the total amount of ammonia obtained during the first period of distillation was considered as preformed ammonia.

Folin (21) 1902-03 devised a method for the determination of ammonia in other animal fluids as well as in urine. By this method 25 cc or 50 cc of the solution of ammonium salt was placed in an aeration cylinder with 1 or 2 grams of sodium carbonate, and 8 to 16 grams of sodium chloride, the latter being used to prevent decomposition. A current of air was driven through the ammonium salt solution, and this carried the ammonia set free by the sodium carbonate into a definite amount of N/10 acid, where it was absorbed. The excess of acid was then titrated and the ammonia calculated.

Shaffer (63) 1903 published a modification of the vacuum distillation method of Boussingault. Shaffer stated, that, although Boussingault had found no decomposition on distilling urea solution in a vacuum with either lime, sodium carbonate or bicarbonate at 50°C, and had concluded that there was no decomposition in urine under the same conditions, there was a slight decomposition in some urines. To shorten the time of distillation to a point where this slight decomposition might be disregarded, methyl alcohol was added to the urine. This lowered the boiling point of the mixture so that a more rapid ebullition took place, and all of the ammonia was driven off by the end of fifteen minutes. Shaffer found the use of sodium carbonate preferable to the use of other alkalies, for with sodium carbonate there was no foaming to render the operation difficult, there was less decomposition than with lime, and no greater than with magnesia, and the expulsion of the ammonia was as rapid when liberated from ammonium salts as from a solution of free ammonia.

The method of vacuum distillation as adapted by Shaffer was as follows: To 50 cc test material were added an excess (15 or 20 grams) of sodium chloride, about 50 cc. of methyl alcohol, and 1 gram of sodium carbonate. The flask containing this mixture was surrounded by a water bath at 50°C. By the use of a water pump the pressure was reduced until the liquid boiled, and the ammonia was driven off into two wash bottles containing N/10 sulphuric acid. At the end of fifteen minutes all ammonia had been given off. The acid was now titrated with N/10 sodium hydroxide, using alizarin red as an indicator. Shaffer stated that this indicator is not affected by the presence of ammonium salts and is very little sensitive to carbonic acid.

Sherman (64) 1905 stated that the Boussingault-Shaffer method for the determination of ammonia in urine had been studied by Berg (3) 1904 with regard to its applicability to the much smaller amounts of ammonia which occur in milk. The following method was used for the determination of ammonia in milk. To 50 cc of the sample were added 50 cc of methyl alcohol, 10 grams of sodium chloride, and 0.5 gram of sodium carbonate. Sherman states that the sodium carbonate liberates the ammonia from its salts, and the sodium chloride diminishes the dissociation of the carbonate, and thus prevents or retards the cleavage of ammonia from the nitrogenous organic compounds in the milk. If the sodium chloride is omitted in the distillation, a larger amount of ammonia is obtained. "For convenience" Sherman says, "the ammonia thus easily split off from organic matter by dilute sodium carbonate at 55°C to 60°C is called cleavage ammonia." "Both the ammonia existing as such in the milk, and the 'cleavage ammonia' increase rapidly under conditions which favor the decomposition of proteids by bacteria or molds."

Folin and Macallum (26) 1912 described a modification of Folin's air current method by which a shortening of the aeration period was made possible by a reduction of the volume of urine used. By this method, a few drops of a solution containing 10 per cent potassium carbonate, and 15 per cent of potassium oxalate were added to 1 to 5 cc of urine, with a few drops of kerosene to prevent foaming. A strong air current was passed through the mixture for 10 minutes or as long as was necessary to drive off all the ammonia. The ammonia was collected in a flask containing 20 cc of water and 2 cc of N/10 acid. At the end of the distillation period the acid was diluted to about 60 cc. One mgm. of nitrogen in the form of ammonium sulphate was diluted to the same volume, and to both solutions were added 5 cc of Nessler's reagent diluted immediately beforehand with about 25 cc of water (Vulte (73) 1915) gives the following method of preparing Nessler's reagent: 35 grams of potassium iodide and 13 grams of mercuric chloride are added to 800 cc water. The mixture is then heated below the boiling point until dissolved, and a cold saturated solution of mercuric chloride is added immediately until the red precipitate formed fails to dissolve after stirring. After the mixture has cooled, 160 grams of potassium hydroxide, dissolved in as little water as possible, are added, and the volume made up to 1 liter. After standing 24 hours, the clear liquid, which should have a pale yellow color, is poured off, and reserved for use.) The relative intensity of the colors of the standard and the unknown ammonium salt solution were then determined by means of a colorimeter. Folin and Macallum state that "a trace of something capable of giving a color with Nessler's solution continues to come long after all ammonia has been removed from urine. This is a point of distinction between urine and ammonium salt solutions."

Kendall and Farmer (37) 1912 determined free ammonia in bacterial cultures by the use of Folin and Macallum's modification of the air current method. They made duplicate determinations of free ammonia on every culture, and found that the greatest discrepancy obtained in their results amounted to 0.35 mgms. of nitrogen per 100 cc. of culture.

Harris (31) 1919-20 used Shaffer's modification of Boussingault's vacuum distillation method for the determination of ammonia in bacterial cultures. Harris, however, omitted the use of sodium chloride, and kept the temperature of the water surrounding the distilling flask at 50°C.

Bushnell (7) 1922, determined ammonia by distillation in the presence of magnesium oxide. He found that when the aeration method was used tests for ammonia could still be obtained with Nessler's reagent after eight hours aeration. (Whether these tests for ammonia were not obtained from the unknown substance of which Folin and Macallum speak may be a matter of conjecture.) In experiments performed with fermenting soils, Bushnell found that in the presence of small amounts of ammonia (10 to 20 mgms. per 100 grams of soil) the distillation method gave 3.6 percent more ammonia than the aeration method. When large amounts of ammonia (60 to 75 mgms. per 100 cc of soil) were present, the aeration method gave 4.0 percent more ammonia than the distillation method.

Kendall (36) states that the determination of free ammonia in bacterial cultures was readily accomplished with the Folin-Macallum modification of the Folin air current method. To 2 cc. of the culture medium were added 1 cc. of an aqueous solution saturated with sodium carbonate and with sodium oxalate, and 1 cc. of kerosene to prevent foaming.

Aeration was continued for fifteen minutes, and the ammonia driven into N/50 hydrochloric acid. The acid, instead of being Nesslerized and compared with a standard ammonium sulphate solution similarly Nesslerized, was titrated with N/50 sodium hydroxide using alizarin as indicator. The results of duplicate determinations were found to check within 0.1 cc of N.50 alkali, or within 0.000,028 grams of nitrogen as ammonia, plus or minus.

De Bord (13) 1923, found that the removal of ammonia with permutit (Folin and Bell (23) 1917) was inapplicable in his work with bacteria because of the fact that the permutit removed variable quantities of amino nitrogen. He found the removal of ammonia by the aeration method of Folin (21) 1902-03 to be accurate under the conditions of his experiments. Secondary octyl alcohol was used to prevent foaming, and aeration was continued for two and one-half hours. De Bord states that this alcohol did not affect the amino nitrogen readings or the ammonia titration.

Amino Nitrogen Determination.

There is a great variation in opinion as to the relative value of the methods in use for the determination of amino nitrogen.

Rice (57) 1915 found the Sörensen titration method (65) 1908 more satisfactory than the Van Slyke method (70) 1912.

Berman and Rettger (2) 1918 employed the formol titration method of Sörensen in conjunction with the biuret test described by Vernon(72)1903.

Harris (31) 1919-20 determined amino acid nitrogen in bacterial cultures from the residue remaining in the distilling flask after the removal of ammonia from a medium. This residue was acidified with acetic

acid, and made to 50 cc volume. Portions of the liquid were used for the determination of amino nitrogen by means of the Van Slyke apparatus.

Denis and Minot (14) 1919 determined amino nitrogen in milk with the Van Slyke micro apparatus. Copper acetate and acetic acid were found to be most satisfactory as protein precipitants in the preparation of the amino solution, since it was possible to obtain clear, rapidly filtering, filtrates without loss of amino nitrogen.

The use of copper salts for precipitating proteins, which are not otherwise easily separated from aqueous solutions was first employed by Ritthausen (59) 1872. Stutzer (67) 1881, also made use of copper salts as protein precipitants and claimed that he obtained complete precipitation of the protein without loss of non protein substances.

Sturgess and Rettger (65) 1922, in addition to the use of the biuret test, and the Sörensen titration method, made amino nitrogen determinations of bacterial cultures by the Van Slyke method. These authors found that, while the Van Slyke method yielded valuable results, its possibilities of variation were so great that they could not place reliance upon a single determination. Duplicate determinations differing by not more than 10 per cent were secured for each amino solution, however. Sturgess and Rettger stated that the difficulties encountered were probably due to the presence of bacterial cells and other suspended matter in the test material. They concluded that the Van Slyke method of amino nitrogen determination was less practical than that of Sörensen because of the extra time and labor required to obtain reliable results.

Bushnell (7) 1922 determined amino acid nitrogen by the Van Slyke method. A preliminary removal of ammonia from the test material was not made, and therefore his results were complicated by the presence of

very large amounts of ammonia. Ammonia corrections were then made as follows: A 2 percent peptone solution containing known amounts of standard ammonium hydroxide was placed in the Van Slyke apparatus, and the amount of nitrogen determined. The difference between the peptone solution alone and a peptone solution plus ammonia was considered to be due to ammonia. Van Slyke stated that about 15 percent of the ammonia nitrogen present would be determined as amino acid nitrogen at 20°C in fifteen minutes. Bushnell found that the percent of ammonia nitrogen recorded as amino nitrogen in five minutes varied from 34.2 percent at 21° to 77.0 percent at 37°C. While his figures were much higher than those of Van Slyke, Bushnell stated that his results gave a fairly uniform curve.

Kendall (36) 1922, in comparing the Sörensen titration method with that of Van Slyke, described Van Slyke's method as being "much more time consuming and presumably somewhat more exact". He concluded, however, that "the precision of the Sörensen titration whenever it can be used in the study of the nitrogenous metabolism of bacterial cultures is commensurate with the accuracy attending the parallel development of bacterial growths."

De Bord (13) 1923, found that he could not obtain control analyses on a sterile 1 percent peptone solution by means of the Van Slyke method. He, therefore, rejected this method for that of Folin (22) 1912. By this method he obtained consistent results in determining amino nitrogen in peptone solutions. On a 1 percent Difco peptone solution, the largest percentage error was 3.5 percent, while in a 2 percent solution the largest percentage error was 5.0 percent.

Determination of Indole and Skatole Production.

Herter and Foster (33) 1906-07 described a method for the separation of indole from skatole, and their quantitative determination. Twenty-five grams of test material are added to 20 cc of water, and 1 to 2cc of a ten per cent solution of sodium hydroxide. This suspension is then subjected to steam distillation, and the resulting distillate is made slightly alkaline with sodium or potassium hydroxide. An excess of β-napthaquinone sodium monosulphonate solution is now added. After a few minutes this substance reacts almost completely with the indole present to form a purplish blue, or blue precipitate. In cases where the concentration of indole is too small to give rise to a precipitate when treated with the napthaquinone compound, the solution simply develops a green or greenish blue color. The solution is now acidified, and subjected to distillation with or without the use of steam. The skatole passes over into the distillate, while the indole is held back in the form of the indole napthaquinone compound. The distillate containing the skatole is now boiled with a solution of Ehrlich's aldehyde in sulphuric acid (5 grams of paradimethylamidobenzaldehyde to 100 cc of 10 percent sulphuric acid.) A purplish blue coloration is produced which , on cooling of the solution becomes a definite blue, soluble in chloroform. Hawk (32) 1917, includes Herter's β-Napthaquinone reaction with indole, and Herter's paradimethylamidobenzaldehyde reaction with skatole among other tests for putrefactive products.

Jordan (34) 1921, states that the production of indole may be determined in Dunham's peptone solution (1 percent peptone and 0.5 percent sodium chloride in water) but that the trypsinized media of Rivas (59) 1912,

and Cannon (8) 1916, are more satisfactory. After four days growth indole may be detected by adding 1 cc of Ehrlich's reagent so that it forms a layer on the surface of the medium. "A red color (due to the formation of a compound soluble in amyl alcohol) indicates a positive result", Jordan states. Ehrlich's reagent is prepared as follows:

Paradimethylamidobenzaldehyde	4 grams
Alcohol 95%	380 cc
Concentrated C.P. HCl	80 cc

Determination of Hydrogen Sulphide and Mercaptan Formation.

Rettger (54) 1906-07 determined hydrogen sulphide and mercaptan formation in the following manner. An equal volume of water was added to 200 cc of the material to be tested in a 500 cc flask. The flask was then collected on the one hand with a bottle containing 3 percent mercuric cyanide, and on the other hand with a wash bottle containing potassium permanganate. The flask was then acidified with 4 to 5 grams of oxalic acid, and warmed for 2 to 3 hours between 30° and 40° C. Hydrogen sulphide came over first and produced a black precipitate while mercaptan produced a yellow precipitate.

Redfield (51) 1912 prepared a special medium for the study of hydrogen sulphide production by bacteria. This medium was composed of 300 grams of Witte's peptone, 75 grams of potassium chloride and 700 cc of boiling water. This mixture was heated to dissolve as much peptone as possible, and the solution was cooled and diluted to one liter. Ten cc of the medium were placed in a flask with 90 cc of the sample under in-

vestigation. The flask was fitted with a ground glass stopper which held lead acetate paper.

Determination of Lactose in Milk.

Folin and Denis (25) 1918 published a method for the determination of lactose in milk. These authors state that their conjecture, that the lactose of milk could be titrated directly without first removing the protein and fat, proved correct.

For cow's milk a dilution of 1:4 (25 cc in a 100 cc flask) was recommended for use. Into a large test tube were introduced 2.8 to 3.4 cc of the diluted milk, 5 cc of a 6 percent copper sulphate solution, a pebble (to prevent bumping) and 4 to 5 grams of a dry salt mixture (made by mixing in powdered form 100 gms. disodium phosphate, 60 grams of anhydrous sodium carbonate, and 30 grams of sodium or potassium sulfocyanate). The tube was then shaken and boiled gently for four minutes before the addition of any more milk, depending on the amount of blue color remaining, were added, and the mixture boiled for one minute. After each addition of milk boiling for one minute was repeated, but the total time of boiling was not allowed to exceed seven minutes.

Folin and Denis stated that 40.4 mgms of anhydrous lactose have the same reducing value as 25 mgms of dextrose. Calculation of the percent of lactose in milk was as follows: 4.04 multiplied by the degree of dilution of the milk and divided by the titration figure gave the percent of lactose in the milk.

Determination of Acid Production

Sörensen(65) 1908, in the course of his studies on enzymes, developed satisfactory colorimetric methods for determining hydrogen ions. Sörensen suggested the use of the symbol p^H for the expression of the hydrogen ion concentration of a medium. Numerically this is the logarithm of the reciprocal of the hydrogen ion concentration. Thus if the latter was 10^{-6} the p^H value would be 6. For the neutral point p^H - 7.0, the number decreasing with increasing acidity, and increasing with alkali concentration. Clark (9) 1915 stated that it had long been recognized that acidity and alkalinity are best defined in terms of hydrogen ion concentration. He further stated that "the rate of enzyme action, the stability of colloidal structures upon which cellular life depends, the solubility of many physiologically important compounds as well as the structure and composition of media, and the color of the indicators used in the adjustment of these reactions - all are dependent in greater or less measure upon hydrogen ion concentrations." Clark pointed out that the adjustment of different media to a "standard reaction" by the method of titration may give widely different hydrogen ion concentrations. He found that some of the difficulties of the older methods of titration were avoided by the use of the hydrogen electrode by means of which the hydrogen ion concentration was determined directly. The potentiometer was found to be more accurate than corresponds to the sensitiveness of bacteria toward changes in the hydrogen ion concentration. Therefore, other methods less time consuming and requiring less elaborate apparatus were devised for bacteriologic work. Clark and Lubs (12) 1917, elaborated the colorimetric method for determining hydrogen ions as developed by Sörensen, and applied them to bacteriologic media.

Dyer (17) 1916, published a method of steam distillation for the determination of the volatile fatty acids. The amount of acid taken for the distillation was first titrated with N/10 potassium hydroxide using phenolphthalein as indicator. The acid was then liberated with an equivalent amount of N/10 sulphuric acid, and distilled with steam while the acid solution was kept at a constant volume. The desired amount of distillate was obtained, and this distillation was titrated to obtain the amount of volatile acids present in the original acid solution.

Wolf and Telfer (81) 1917 determined volatile and non-volatile acid production of anaerobic bacteria as follows: "One hundred cc of the fermentation liquid were acidified with normal sulphuric acid, and exhaustively steam distilled. The distillate was titrated with decinormal sodium hydrate, using phenolphthalein as an indicator, and the total amount of volatile acid present ascertained. The residue in the distilling flask, containing the non-volatile portion, was transferred to a liquid extraction apparatus, and the acids dissolved out with ether. This operation was a continuous one and extended over a period of forty-eight hours. The ethereal solution was then evaporated to dryness, and the residue dissolved in water. The solution containing the acids was then filtered, and titrated with deci-normal sodium hydrate."

Wolf and Harris (79)(80) 1918-19 and Harris (31) 1919-20, determined the hydrogen ion concentration of various media, and of cultures of anaerobic bacteria by comparison with a set of phosphate standards containing phenol sulphonephthalein as indicator.

Wolf (76,77,78) 1918-19, and Harris (30) 1919-20, determined volatile acid production by titration against N/10 alkali using phenolphthalein as indicator.

Identification of Volatile Fatty Acids.

Agulhon (1) 1913, proposed colorimetric tests for the identification of the fatty acids. Dyer (17) 1916, after a study of the solubility of various metallic salts of the volatile fatty acids in various organic solvents, found that only the salts of iron and copper respond satisfactorily to tests of this nature. He, therefore, elaborated a number of qualitative color tests by which the presence of certain individual members of the volatile acid series could be detected. These colorimetric qualitative tests depend upon the difference in solubility of the iron and copper salts of the acids in various organic solvents. Copper chloride of a strength approximating 0.1 normal, and iron chloride in solution between 2 and 3 percent in strength were used as the precipitants in these tests, and amyl alcohol, ether, gasoline, kerosene, petrol ether, and ethyl acetate free from alcohol as solvents.

Dyer states that as no individual colorimetric test for formic acid could be found, it is necessary to make use of its reducing properties to identify it, for example, the formation of a blue color with nitric acid and bichromate of potash mixture as suggested by Agulhon.

The presence of acetic acid was recognized by the use of turpentine as solvent and copper chloride as precipitant. The copper salt of acetic acid was dissolved by the turpentine, and brought to the surface of the solution as a deep greenish blue layer.

Propionic acid was identified by the use of amyl alcohol as solvent, and iron chloride as precipitant. A positive test was indicated by a pronounced yellow tinge of the amyl alcohol, and a collection of brown insoluble salts at the junction of the two liquids while the aqueous solution was colorless.

Butyric acid was identified by the use of amyl alcohol, or ether, as solvent and iron chloride as the precipitant. In amyl alcohol, the iron salt of butyric acid gave an intense red color. If ether was used as the solvent, the two liquids emulsified somewhat upon agitation, and the other layer remained colorless, while a collection of insoluble salts formed at the junction of the two liquids.

Valeric acid was also recognized by the use of ether as solvent, and iron chloride as precipitant. A positive test was indicated by a reddish yellow color of the ether, and a collection of insoluble salts at the junction of the aqueous solution and the ether layer.

Caproic acid was identified by the use of gasoline, kerosene, or petrol ether as solvent, and copper chloride as precipitant, or by the use of ethyl acetate free from alcohol as the solvent, and iron chloride as precipitant. The copper salt of caproic acid gave a decided blue color to the solvent, while the iron salt of the caproic acid colored the ethyl acetate a rich iron red.

The following methods were selected for the determination of the changes produced in the composition of milk by aerobic spore-bearing bacteria:

Ammonia determination - Shaffer's method

Amino nitrogen determination (Wolf and Harris' method
(Denis and Minot's method

Lactose determination - Folin and Denis' method

Total acidity - Hawk's method

Volatile acidity - Dyer's method.

EXPERIMENTAL WORK

A. Source of material.

As a preliminary to the quantitative determination of the decomposition products formed in milk by aerobic spore-bearing bacilli, a study of the flora of pasteurized milk was carried out. Samples of pasteurized milk, varying in amount from 300 to 400 cc were placed in water baths, and subjected to 70°C and 80°C, for thirty minutes. At the end of this time, the flasks of milk were placed in the incubator at 37°C. and allowed to remain for four or five days. The milk was then plated, and the plates were incubated for forty-eight hours, or more, at 37°C.

The aerobic spore bearing bacteria which were isolated were identified according to recent investigations. The following spore bearing bacilli were isolated from Baltimore milk: Bacillus cereus, Bacillus albolactus, Bacillus mesentericus vulgatus, Bacillus mesentericus fuscus, Bacillus subtilis viscosus, Bacillus simplex and Bacillus circulans.

A sample of raw milk from Fort McCoy, Florida, was subjected to 80° for thirty minutes. The following additional organisms were isolated: Bacillus megatherium, and a spore bearing bacillus resembling Bacillus brevis, as described by Ford, in its morphology and in some of its cultural reactions. Contrary to the production of alkalinity in glucose, lactose and saccharose broth as described in cultures of Bacillus brevis, this organism forms slight amounts of acid in sugar broths. Milk is softly coagulated on the eighth day. Milk cultures of this bacillus have an unpleasant odor resembling that of codfish.

A gas producing aerobic spore bearing bacillus was also isolated from the samples of Florida milk, and was identified as Bacillus asterosporous.of Myer.

Of the aerobic spore bearing bacilli isolated from milk, only those which digest milk were used in the later chemical work. The organisms selected for chemical investigation were Bacillus cereus, Bacillus albolactus, Bacillus mesentericus vulgatus, Bacillus mesentericus fuscus, Bacillus megatherium, Bacillus simplex, Bacillus subtilis viscosus, and Bacillus brevis. The same strain of each bacillus was used throughout the series of analyses of the decomposition products formed in milk by the action of this bacillus.

One cubic centimeter of a plain milk culture of the bacillus under study, was introduced into a liter flask containing 400 cc of sterile milk. The flask was then incubated at 37°C. for eight days, or for twenty days.

In Experiment I to XVII a liter flask containing 400 cc of sterile milk of the same sample, was incubated with the culture for the same length of time, and analyzed on the same day. By this means both sterile milk and culture were subjected to exactly the same conditions in regard to temperature and evaporation. In Experiment XVIII to XXXIX, inclusive, four or five flasks were prepared from the same well mixed sample of milk, and placed on ice until needed. Of these flasks, one was analyzed when sterile, and served as a control for the remaining flasks, which were inoculated and incubated at different times. In these later experiments, therefore, the sterile milk and cultures were not subjected to the same conditions as to temperature and evaporation. However, the error introduced by the difference in the amount of evaporation from the small number

of flasks was not thought sufficient to warrant the increased labor of analyzing a sample of sterile milk for each culture studied, as was done in the earlier experiments.

The following changes produced in milk by the action of the bacteria were determined:

(1) Change in ammonia content.

(2) Change in amino nitrogen content.

(3) Change in lactose content.

(4) Change in hydrogen ion concentration.

(5) Change in volatile acid content.

B. Methods used

Determination of Ammonia Content of Sterile Milk and Milk Cultures of Aerobic Spore Bearers.

In determining the ammonia content of sterile milk and milk cultures of the aerobic spore bearing bacilli under study, the vacuum distillation method as described by Shaffer (63) 1903 was used. To 25 cc of the medium were added 40 cc of methyl alcohol, and 1 gram of sodium carbonate. Under reduced pressure obtained with a water pump, the ammonia was distilled into two wash bottles containing respectively 25 cc and 10 cc of N/10 sulphuric acid plus 10 cc of distilled water. At the end of fifteen minutes' distillation, the acid was titrated with N/10 potassium hydroxide. Alizarin red was used as the indicator in the titrations, for Shaffer states that it is not affected by the presence of ammonium salts, and is very little sensitive to carbonic acid. Each distillation was duplicated, and the average of the two final titration figures was used for the calculation of milligrams of nitrogen as ammonia per 100 cc of the medium. The calculations of milligrams of nitrogen as ammonia per 100 cc of the medium were made as follows:

10 cc. of the total volume of acid were neutralized by 6 cc. of N/10 KOH

55 cc. (total volume of acid) were neutralized by 33 cc. N/10 KOH

55 cc. - 35 cc. N/10 H_2SO_4

35 cc. N/10 H_2SO_4 -- 33 cc. N/10 KOH = 2 cc N/10 NH_3

2 x 1.4 (mgms. N. in 1 cc. N/10 NH_3) = 2.8 mgms. in 25 cc of milk.

2.8 x 4 = 11.2 mgms. N as ammonia per 100 cc. of milk.

Amino Nitrogen.

In the determination of amino nitrogen in sterile milk and milk cultures, the Van Slyke method was used. Two methods of preparing the amino solution were used as a means of placing a check on the technique of the operation of the Van Slyke apparatus. Following the method described by Harris (31) 1919-20, the residue, left in the distilling flask after the distillation of ammonia, was acidified with acetic acid and filtered. The filtrate was then made up to 50 cc, and 1 cc of this amino solution was used for the determination of amino nitrogen in the Van Slyke micro apparatus. The calculation of milligrams of amino nitrogen per 100 cc of the medium was then made as follows:

1 cc of amino solution = 1/2 cc of medium
1/2 cc = .23 cc nitrogen (reading of gas burette)
100 cc medium = 46 cc nitrogen.
46 x .5520 (mgms. of amino nitrogen corresponding to 1 cc of nitrogen at 27°C, and 760 mm. pressure =
29.39 mgms. of amino nitrogen per 100 cc of medium

Duplicate solutions were prepared according to Harris' method, and duplicate determinations were made of each solution. The largest percentage error accepted from the same solution, or from the average of the determinations from the two solutions was 10 percent. The second method used was that devised by Denis and Minot (14) 1919 for the determination of amino nitrogen in milk. Twenty cc of milk or culture were placed in a 200 cc volumetric flask. To the medium were added 40 cc of N/100 acetic acid, 10 cc of 5 percent copper acetate solution, and 60 cc of distilled water. The flask was placed in a boiling water bath for 30 minutes and at the end of that time, 1 cc of a 15 percent solution of potassium oxalate was added. The mixture was cooled, brought to volume, and filtered through a dry filter paper. To the filtrate was now added 0.5 grams of

powdered potassium oxalate, and the solution allowed to stand for at least an hour before filtering. Fifty cc of the final filtrate were evaporated on a water bath to 1 to 2 cc and this concentrated amino solution was used for the determination of amino nitrogen by means of the Van Slyke apparatus. The calculation of milligrams of amino nitrogen per 100 cc of the medium was as follows:

50 cc filtrate = 5 cc medium.
5 cc medium = .3cc nitrogen (reading of gas burette)
.3 x 20 = 6 cc (amount nitrogen in 100 cc of the medium)
6 x .5520 (milligrams of amino nitrogen corresponding to 1 cc of nitrogen at 27°C and 760 mm pressure
=
3.31 (mgms. of amino nitrogen per 100 cc of the medium)

Duplicate solutions were made according to this method and duplicate determinations made of each solution. As in the solutions prepared by Harris' method, the largest percentage error accepted was 10 percent.

Lactose

In determining the lactose content of sterile milk and milk cultures of aerobic spore bearing bacilli, the method used was that devised by Folin and Denis (25) 1918 for the determination of lactose in milk. Twenty-five cc of sterile milk or milk culture were made up to 100 cc with distilled water. Into a large test tube were introduced 2.8 to 3.4 cc of the diluted medium, 5 cc of a 6 percent copper sulphate and 4 grams of a dry salt mixture (composed of 100 grams of disodium phosphate, 60 grams of anhydrous sodium carbonate, and 30 grams of sodium sulfocyanate). The mixture was now boiled gently for 4 minutes. At the end of this time, an amount of milk, varying from 0.05 cc to 0.1cc as indicated by the intensity of blue color remaining, was added. After each addition of milk the mixture

was boiled for 1 minute. The total boiling period was not allowed to exceed 7 minutes. The percentage of lactose was calculated as follows:

$$\frac{4.04 \times 4 \text{ (degree of dilution)}}{3.8 \text{ (cc of milk added)}} = 4.25 \text{ percent}$$

Denis states that this quantitative sugar method, as well as any other titration method, will give reduction not only with lactose but with any other reducing bodies present, and that it is possible that the action of bacteria on either protein or lactose might give rise to reducing bodies such as aldehydes, ketones and acids. In recording the results obtained by the titrations of milk cultures of bacteria, therefore, they have been designated as reducing substances calculated as percent of lactose.

Hydrogen ion concentration.

The hydrogen ion concentration of each sample of sterile milk, and of each bacterial culture was determined colorimetrically by comparison with sets of standard solutions containing phenol-sulphonephthalein and brom cresol purple as indicators. A 1-10 dilution of the medium was prepared, and to 2-1/2 cc of the diluted medium was added .2 cc of indicator. The diluted medium plus the indicator was then compared with the standard solutions for the determination of the p^H value.

Total Acidity.

Total acidity was determined by titration of a 1-10 dilution of the medium with N/10 potassium hydroxide using phenolphthalein as an indicator.

Volatile Acids.

The presence of volatile acids in sterile milk and milk cultures was determined by the steam distillation method of Dyer (17) 1916 as modified from that of Duclaux (16) 1900. One hundred cc of milk, or culture, were made acid to Congo red paper, and steam distilled. The distillation was carried out at a uniform rate for each experiment, and the same amount of distillate collected (500 cc) Harris (31) 1919-20 stated that except for acids of low volatility, such as formic acid, this method would yield from 90 percent to 95 percent of the total acid present. The distillate was then titrated with N/10 potassium hydroxide, using phenolphthalein as an indicator. As the amounts of volatile acids in milk, and the amounts formed by the aerobic spore bearing bacilli were very small, one cc pipettes were used for the titrations. Results were expressed as cc of N/10 potassium hydroxide per 100 cc of the medium.

In the identification of the volatile acids in milk, and milk cultures of the bacteria studied, Dyer's qualitative color tests were used. One hundred cc of the distillate obtained from the steam distillation of the medium were neutralized with potassium hydroxide, and concentrated to about 20 cc. Portions of this concentrated distillate were used for tests for the presence of formic, acetic, butyric, valeric and caproic acid.

Indole, Hydrogen sulphide, and Mercaptan.

Tests for indole were made with Ehrlich's reagent - an alcoholic solution of para-dimethylamidobenzaldehyde plus hydrochloric acid.

Hydrogen sulphide and mercaptan formation were tested for by the method given by Hawk (32) 1917. In the culture flask was inserted a cork furnished with a glass tube to which was attached a wash bottle containing a 3 percent solution of mercuric cyanide. The culture was then incubated for thirty days at 37° C.

C. Personal Investigations of Sterilised Milk.

Ammonia.

The Ammonia content of sterile milk ranged from 86.24 to 125.4 mgms. per 100 cc of the medium. While these figures are much higher than those given by Kendall, Day and Walker, Table III, this increase may be partially explained by the fact that all milk used in these investigations was commercial milk obtained from a dairy at least thirty hours after milking. Richmond (58) 1914 says that the presence of ammonia in milk may be due to bacterial action. It is evident that the amounts of ammonia present in milk of the age used would vary greatly depending upon the bacteria present.

Amino nitrogen.

On comparison of the results obtained from the determination of amino nitrogen from solutions prepared according to the method of Wolf and Harris with results obtained by the method of Denis and Minot, it was observed that the actual amount of amino nitrogen in a sample of milk or culture was much larger when determined from a solution prepared by the former method than when prepared according to the latter method.

When determined from solutions prepared by Harris' method, the amino nitrogen of sterilized milk varied from 10.17 to 46.27 milligrams per 100 cc of the medium. The amounts of amino nitrogen in sterile milk as determined from solution prepared by the method of Denis and Minot ranged from 1.8 to 4.09 mgms. per 100 cc of the medium. In Table I are given the amounts of amino nitrogen in milligrams per 100 cc of the medium for the twenty-seven samples of sterile milk analyzed.

Several possible sources of error may be pointed out in Harris' method. Folin and Denis (24) 1912, found that certain nitrogenous substances, such as creatine, asparagine, and tyrosine, when added to blood could not be recovered quantitatively after precipitation with methyl alcohol.

Greenwald (30) 1915, concluded that alcohol precipitates some nitrogenous non-protein constituents of the blood, of which 25 to 50 per cent represents amino acid nitrogen as determined by Van Slyke's method. Bock (5) 1916, found that amino acids added to blood could not be recovered quantitatively after the use of methyl alcohol as a protein precipitant.

A second source of error is indicated by Van Slyke (70) 1912. He states that it is essential that all the ethyl alcohol, added to the medium before the ammonia determination, should be driven off, for it decomposes the nitrous acid in the deaminizing vessel of the Van Slyke apparatus with the formation of large volumes of gases, which can be removed with permanganate only with difficulty, and by the use of perfectly fresh permanganate solution.

A third source of error lies in the hydrolysis of the protein of the milk and milk cultures by hydrolysis with an alkali. In a communication from Dr. Denis, she states that the Harris method of preparing the amino solution would undoubtedly yield high results owing to the distillation of milk in the presence of sodium carbonate.

It is therefore evident that while the amount of amino nitrogen in milk or culture might be decreased by precipitation with methyl alcohol, the amount might also be increased by hydrolysis of the proteins during the removal of ammonia, and by incomplete removal of the alcohol during this process. That there was a constant increase in the amino nitrogen of sterile milk when the amino solution was prepared by the method of Harris is shown in Table I. That the amounts of amino nitrogen formed by the aerobic spore bearers studied were usually larger when determined from a solution prepared by Harris' method is shown in Tables IV to XI, inclusive. This increase in amount of amino nitrogen formed was probably due to the fact that the proteins of the milk cultures were so altered by bacterial action as to be more readily hydrolyzed than the proteins of sterile milk.

Lactose.

The percentage of lactose in the twenty-seven samples of sterile milk analyzed ranged from 2.93 percent to 5.29 percent. As will be shown in the review of the previous investigations of the composition of milk, the lactose content of milk has been proved to vary from 3.5 percent to 5.0 percent.

Hydrogen Ion Concentration.

The hydrogen ion concentration of sterile milk varied in the twenty-seven samples from 6.4 p^H to 6.6 p^H. A 1-10 dilution of milk was used in the determination of hydrogen ion concentration. Clark (10) 1921, states that a well buffered solution may often be diluted without seriously altering the p^H. "When dealing with complex solutions which are mixtures of very weakly dissociated acids and bases and their salts, and especially when the solution is already near neutrality, dilution has a very small effect on p^H." "Differenceswhich may be observed are quite as likely to be due to the changes in the protein and salt content."

Total acidity

The total acidity of sterilized milk was determined by titration of a 1-10 dilution with N/10 potassium hydroxide, using phenolphthalein as an indicator. From 1.2 to 3.2 cc of N/10 potassium hydroxide were required to neutralize 100 cc of the solution. This gave a total acidity of 12 cc to 32 cc of the undiluted milk.

Volatile Acid Content.

Volatile acid content was determined by titration of the distillate from 100 cc of the milk with N/10 potassium hydroxide, using phenolphthalein as an indicator. As stated in the discussion of methods, Harris (31) 1919-20 estimated that this would give from 90 percent to 95 percent of the total acid present, except in the case of acids of low volatility such as formic acid. The volatile acid content of the twenty-seven samples of sterile milk analyzed, as determined from 500 cc of distillate obtained by the steam distillation of 100 cc of milk, ranged from 0.7 to 2.0 cc of N/10 potassium hydroxide required to neutralize 100 cc of the medium. While these figures are much lower than those given by Wolf and Harris as shown in Table I (13.6 cc to 16.0 cc) they are comparable to the amount (2.52 cc) of N/10 potassium hydroxide, which Bushnell found neutralized 100 cc of sterile milk.

Acetic acid, butyric acid and caproic acid were identified in the volatile acids of sterile milk by means of Dyer's colorimetric tests.

A reduction of dilute solutions of potassium permanganate was observed in a number of instances. As this was one of the tests used by Rettger (52) for the recognition of the volatile sulphide given off when milk is heated above 85°, a positive test could not be taken as proving the presence of formic acid in milk.

A volatile sulphide which blackened lead acetate paper was found to be given off from all samples of acidified or unacidified milk when subjected to steam distillation.

Indole

The distillate obtained by the steam distillation of acidified milk gave a positive test for indole when tested with Ehrlich's reagent. The distillate of unacidified milk failed to give this test, however.

As stated in the description of the composition of milk one of the products of the hydrolysis of casein is tryptophane or indol-amino-propionic acid. Osborne and Mendel (48) 1915, have shown that tryptophane is present in maximum amount in lactalbumin.

Plimmer (49) 1918, stated that tryptophane forms a red color with para-dimethylamidobenzaldehyde. It is indicated, therefore, that the positive test obtained from the distillate of acidified milk merely denoted the production of tryptophane by acid hydrolysis of the milk.

TABLE I.
STERILE MILK.

Sample	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Nitrogen as Ammonia	11.2	35.2	11.2	15.79	18.92	15.79	54.32	26.30	11.2	38.64	25.59	32.76	17.36
Amino Nitrogen (Wolf & Harris		14.31		10.60	23.84	46.27	10.17	13.35	42.63	33.2	28.40	24.09	21.80
Amino Nitrogen (Denis & Minot		2.33	1.88	2.55	3.07	3.12	1.80	2.03	2.79	4.09	2.4	2.37	2.59
Reducing Substances	2.93	4.25	4.04	4.25	4.48	4.14	4.36	4.14	4.19	4.36	3.67	3.94	3.94
P^H	6.6	6.6	6.6	6.5	6.6	6.6	6.6	6.5	6.6	6.6	6.8	6.4	6.4
Total Acidity (1-10 dil)	2.2	1.2	1.6	1.2	1.6	1.8	1.2	1.6	1.6	1.2	1.6	1.6	2.0
Volatile Acids	1.0	1.0	1.0	0.8	1.0	1.0	0.7	1.0	1.0	1.0	1.0	1.0	1.0

Milligrams per 100 cc of the medium
Calculated as percentage of lactose
Cubic centimeters of N/10 KOH required to neutralize 100 cc of the medium

TABLE I (cont.)

STERILE MILK

XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII	XXIV	XXV	XXVI	XXVII
17.36	18.87	32.48	46.59	26.32	23.52	11.2	38.64	23.52	29.63	32.48	20.72	26.60	26.60
28.10	29.42	31.73	24.03	25.04	21.29		20.39	20.27	31.23	22.30	17.56	45.53	30.60
2.61	2.54	2.32	2.40	3.49	2.30	2.43	3.23	3.91	3.06	3.37	2.10	2.50	2.10
3.36	4.48		4.25	4.09	3.89	4.14	4.19	5.13	5.47	5.29	3.55	3.99	4.25
6.5	6.4	6.6	6.6	6.4	6.5	6.6	6.6	6.4	6.6	6.4	6.4	6.4	6.4
1.6	1.8	1.8	1.8	2.0	2.2	1.8	2.0	2.8	3.2	2.6	2.0	2.0	3.2
1.0	1.4	1.4	1.6	1.8	1.6	1.6	1.2	1.6	1.6	1.6	1.4	1.2	2.0

TABLE II

RESULTS OF INVESTIGATIONS ON PRODUCTS FORMED BY PURE CULTURES OF AEROBIC SPORE BEARING ORGANISMS.

	Reactions on litmus milk	Fermentation of lactose	Fermentation of dextrose	Production of indol in milk	Liquefaction of gelatin
Bacillus cereus	-	-	+	+	+
Bacillus albolactus	+	+	+	-	+
Bacillus megatherium	-	-	+	-	+
Bacillus mesentericus fuscus	-	-	+	-	+
Bacillus mesentericus vulgatus	-	-	+	-	+
Bacillus simplex	-	-	+	-	+
Bacillus brevis	+	+	+	+	+

D. Previous Investigations of the

Composition of Milk.

Proteins.

Richmond (58) 1914 stated that of the various proteins described as existing in milk the four of whose existence we have the strongest evidence are casein, lact-albumin, lacto-globulin, and Storch's muccoid. Storch claimed that the fat globules of milk are surrounded by a gelatinous muccoid membrane.

Van Slyke (71) stated that the number of proteins in milk reported by different investigators varied from one to eight. The weight of evidence, he states, justified the belief that fresh normal milk contains three distinct protein compounds, viz., calcium casein, lact-albumin, and lacto-globulin. "The multiplicity of milk proteins described in literature is largely due to the action of reagents or ferments on milk, forming a variety of compounds, usually in the nature of decomposition products, which do not exist in fresh normal milk."

Van Slyke stated that in fresh normal milk the percentages of proteins will commonly be found within the following limits:

Total proteins.....	2.50 to 3.75%
Casein.....	2.00 to 3.00%
Albumin and globulin.....	0.45 to 0.90%

Casein is a complex chemical compound belonging to the phosphoproteins. Van Slyke states that our present knowledge of the structure of any protein is stated by giving the percentages of the different amino acids formed by hydrolysis of the protein.

The products of the hydrolysis of casein have been given by Osborne and Guest (47) 1911 and are as follows:

	%
Glycocoll or glycine (amino-acetic acid)	0.00
Alanine (a-amino-propionic acid)	1.50
Valine (a-amino-isovaleric acid	7.20
Leucine (a-amino-caproic acid)	9.35
Proline (pyrrolidine-carboxylic acid)	6.70
Phenylalanine (phenyl-a-amino propionic acid	3.20
Glutaminic acid (amino-glutaric acid)	15.55
Aspartic acid (amino-succinic acid)	1.39
Cystine (amino-thiolactic acid)	
Serine . (a-amino-B-hydroxy-propionic acid)	0.50
Tyrosine (oxyphenyl-a-amino-propionic acid)	4.50
Oxyproline	0.23
Histidine (a-amino-B-imidazol-propionic acid)	2.50
Arginine (a-guanidino-a-amino-valeric acid)	3.81
Lysine (a,e, diamino-n-caproic acid)	5.95
Tryptophane (indol-amino-propionic acid)	1.50
Diamino-trioxy-dodecanic acid, structure unknown	0.75
Ammonia	1.61
Sulphur	0.76
Phosphorus	0.85

Van Slyke states that in what form of combinations sulphur and phosphorous are present in casein we have little information as yet.

Lact-albumin differs from casein in composition and properties. It contains no phosphorous, but about twice as much sulphur as casein.

Lacto-globulin, Van Slyke states, is present in the normal milk of cows in only minute amounts. It closely resembles serum globulin in general properties.

Rettger (52) 1902 found that on heating normal milk above 85°cc a partial decomposition of the milk proteids occurred, which was indicated by the liberation of "a volatile sulphide probably hydrogen sulphide." The amount of sulphide given off was sufficient to blacken lead acetate paper, and to decolorize dilute solutions of potassium permanganate. Alkalis and alkaline reacting phosphates facilitated this decomposition, while acids and acid reacting phosphates retarded it.

Meigs and Marsh (45) 1913 found a constituent of milk, which forms a large part of the unknown alcohol substances, and which gives a strong test for unoxidized sulphur.

Ammonia and Amino Nitrogen.

In Table III are given the milligrams of ammonia nitrogen per 100 cc of sterile milk as given by Wolf and Harris (79) 1916-17, Wolf (76) 1918-19, and Kendall, Day and Walker (41) 1922. The ammonia content was determined in Wolf and Harris' work by the vacuum distillation method of Shaffer, and in Kendall, Day and Walker's work by the Folin-Macallum modification of the Folin air current method. The results given by the various workers range from 2.8 mgms. of ammonia nitrogen per 100 cc of medium to 15.1 mgms. per 100 cc of medium. On the whole, however, the figures given are fairly constant.

The amounts of amino nitrogen per 100 cc of medium are given as determined by Wolf and Harris, Kendall, Day and Walker, and by Denis and Minot (14) 1919. In both Wolf and Harris, and Denis and Minot's work, the amino nitrogen was determined by the Van Slyke apparatus, but the method of preparing the amino solution differed. Kendall, Day and Walker determined amino nitrogen by the Sörensen titration method, and, as shown in Table III, their results while consistent , are larger than those of the other authors. We may assume that the milk used by Wolf and Harris, and by Kendall, Day and Walker had been autoclaved before the analyses were made, while that analyzed by Denis and Minot had not been so treated. The results given by Wolf and Harris, and by Denis and Minot were more comparable however, than those of Kendall, Day and Walker with the other authors. It seems possible that the method used in determining the

amino nitrogen may play a part in these results, for the total results given by the various workers as shown in Table III, range from 2.6 mgms. to 25.2 mgms. of amino nitrogen per 100 cc of the medium.

Hawk (32) 1917 gave the principal solids of milk as olein, palmitin, stearin, butyrin, casein, lact-albumin, lacto-globulin, lactose, phosphates of calcium, potassium and magnesium, citrates of sodium, and potassium, and chloride of calcium. He stated that milk also contains some iron, and at least traces of lecithin, cholestrol, urea, creatine, creatinine, and the tri-glycerides of caproic, lauric and myristic acids.

Lactose.

Hawk (32) 1917 gave the "sugar" content of cow's milk as ranging from 3.5 per cent to 5.0 per cent. Plimmer (49) 1918 gave the lactose of cow's milk as 4.8 percent. Wolf and Harris (80) 1918 found the lactose of twelve samples of sterile milk to vary from 3.68 percent to 4.57 percent.

Total Acids.

Richmond (58) 1914, stated that the acidity of milk to phenolphthalein is due partly to the mono and dibasic phosphates, and partly to the dissolved carbonic acid.

Richmond stated that Grunzweig has proved that the butyric acid of the fat of milk is normal, and that Koefoed has proved that the caproic acid of the fat of milk is normal. Bechamp, he stated, has described the salts of acetic acid in milk. In freshly drawn milk lactic acid is in all probability absent, but, as almost immediately after milking, milk contains organisms which produce lactic acid, it may be considered as a normal constituent of milk. Richmond stated, however, that lactic acid probably exists, not

in the free state, but as a salt - at all events until the acidity is sufficient to curdle the milk on boiling. "The view of Bechamp, that lactic acid is produced from milk by organisms derived from the udder, was shown to be erroneous by the fact that Lister, Pohl, Warington, and others, succeeded in preserving milk drawn into sterile vessels for a considerable length of time."

Matthews (43) makes the statement that citric acid may be present in cow's and human milk to the extent of .237 percent. He also stated that butter yields about 7 percent of volatile fatty acids, which are mainly butyric and caproic acids. There are also small amounts of caprylic and capric acids.

The total acidity of sterile milk as shown by Wolf and Harris, and by Kendall, Day and Walker varied from an acidity requiring 10 cc of N/10 alkali to neutralize 100 cc of medium to an acidity neutralized by 32 cc of N/10 alkali per 100 cc of medium.

Wolf (76) gives the volatile acid content of three samples of sterile milk as follows: 10.4, 13.6, and 16.0 cc of N/10 alkali were required to neutralize 100 cc of the medium. Bushnell (7) found the volatile acid of one sample of sterile milk to be neutralized by 2.52 cc of N/10 alkali per 100 cc of the medium.

TABLE III

COMPOSITION OF MILK.

Author	Table	Mgms. of N. as ammonia per 100 cc. of Med.	Mgms. of Amino N per 100cc of medium	cc. of N/10 alkali per 100 cc of medium
Kendall, Day & Walker	I.B. Welchii	4.2	22.4	14.10
" " "	" "	5.6	14.7	11.0
" " "	" "	4.2	25.2	17.0
" " "	II." "	7.0	21.0	12.9
" " "	" "	2.8	22.4	14.0
" " "	" "	4.2	21.0	14.0
" " "	" "	4.9	22.4	12.0
"	" "	3.5	22.4	14.0
"	" "	7.7	23.1	14.0
"	I.Vibrion Septique	6.3	15.9	17.0
"	" "	5.6	18.2	18.0
"	" "	5.6	18.2	16.0
"	" "	4.9	15.9	17.0
"	" "	4.2	21.0	17.0
"	" "	5.6	22.4	15.0
"	I. B.fallax	1.2	22.4	11.0
"	" "	7.7	23.1	14.0
"	" "	2.8	21.0	32.0
"	I. B. tertius	7.0	21.0	12.0
"	I. B. tetanus	5.6	21.0	17.0
"	" "	7.0	21.0	14.0
"	" "	5.6	22.4	10.0
"	T.B. pseudo tetanus	6.3	18.4	15.0
"	T.B. XxX.botulimun	8.4	16.8	14.0
"	" "	5.6	20.2	15 .0
"	" "	5.6	18.2	16.0
"	" "	4.2	21.0	17.0
"	T.B. bifermans	7.0	22.4	10.0
"	" "	4.9	21.7	13.0
"	I.B. oedamatiens	4.9	22.4	12.0
"	" "	7;7	23.1	14.0
"	T.B. aerofitidis	3.5	21.7	11.0
"	I.B. sporogenes	7.0	13.6	15.0
"	" "	6.3	18.9	15. 0
"	I.B. histolyticus	5.0	19.6	14.0
"	" "	3.5	21.7	12.0
Wolf & Harris	XIV.B.sporogenes	4.4	5.0	15.0
"	" "	7.4	7.0	15.0
"	" "	8.0	8.0	13.0
"	" "	4.5	7.7	
"	VIII B.Welchii		5.7	15.0
"	" "		5.0	15.0
"	" "		4.4	15.0
"	" "		5.3	
"	" "	4.0	8.5	12.0
"	I.B. histolyticus	3.0	5.0	
"	" "	8.0	13.0	
Wolf	I. Vibrion septique	3.5	9.0	
"	I.B.proteus	9.8	17.1	
"	"	13.1	13.8	
Denis & Minot			3.03	
"			.13	
"			4.30	

TABLE XII (cont.)
COMPOSITION OF MILK

AUTHOR	Table	Mgms of N. as ammonia per 100 cc of medium	Mgms of Amino N per 100 cc of medium	cc of N/10 alkali per 100 cc of medium
Denis & Minot			4.18	
"			5.1	
"			3.5	
"			3.2	
"			2.6	
"			4.4	
"			3.6	
"			4.8	
"			4.9	
"			7.3	
"			5.3	
"			4.7	
"			3.1	
"			7.4	
"			7.0	
"			8.0	
"			3.3	
"			3.68	
"			4.64	
"			3.46	
"			7.8	
"			7.7	
"			5.1	
"			5.4	

Kendall, Day and Walker used theFolin MacCallum modification of the Folin air current method for determining ammonia; the Sörensen titration method for determining amino nitrogen, and used neutral red as indicators in titrating total acidity.
Wolf and Harris determined ammonia byShaffer's vacuum distillation method, and amino nitrogen by Van Slyke's apparatus.
Denis and Minot determined amino nitrogen by Van Slyke's apparatus.

E. Results of chemical investigations of the products of pure cultures of aerobic spore-bearing organisms

(1) Bacillus cereus.

In Table IV are shown the changes produced in the ammonia, amino acid, lactose and the total and volatile acid content of milk by Bacillus cereus in eight days, and in twenty days. Each of the seven analyses were made at different times, but conditions differed only in the age of the strain, and in the age of the seed culture.

Ammonia and Amino nitrogen Formation.

A constant progressive increase in ammonia formation and in amino nitrogen formation was observed. The rise in nitrogen as ammonia varied from 3.92 mgms. to 6.72 mgms. in eight days. In twenty days an increase of nitrogen as ammonia of 12.32 mgms. per 100 cc of medium was found in each of the two twenty-day cultures analyzed.

Amino nitrogen formation in eight days varied from 23.67 mgms. to 38 mgms. per 100 cc. (Denis and Minot's method), and from 24.76 mgms. to 36.69 mgms. per 100 cc as determined by the method of Wolf and Harris. In twenty days there was an increase of amino nitrogen varying from 57.78 mgms. to 60.83 mgms. per 100 cc of the medium (Denis and Minot) while the amount formed as given by the method of Wolf and Harris was 85.27 mgms. per 100 cc of the medium.

Reducing substances.

Reducing substances calculated as lactose increased in eight days in amounts varying from 0.11 percent to 0.72 percent, but in twenty days a decrease of 0.7 and 0.11 percent was shown. As stated in the discussion of methods, any reducing bodies formed by the action of the bacteria would be recorded in the determination of lactose.

Total acidity.

The total acidity as shown by titration, or "the reaction", showed an increase varying from 2.0cc to 3.4 cc per 100 cc of the medium in eight days, and an increase varying from 2.0 cc to 7.0cc per 100 cc of the medium in twenty days.

The hydrogen ion concentration, on the contrary, showed a decrease of 0.3 in eight days, and in twenty days a decrease of 1.0 and 1.2

Kendall, Day and Walker (40) 1913, in describing the action of Bacillus proteus on milk, stated that the action became progressively acid in spite of the rapid peptonization. They state "this might be attributable to the liberation of acid phosphates following the decomposition of the casein, or to a partial decomposition of the butter fats with the liberation of fatty acids."

Clark and Lubs (11) 1917, in commenting on the findings of Kendall, Day and Walker, explain that these authors were speaking of the "reaction as determined by titration. Clark and Lubs state that "an increase in this value is not at all incompatible with a decrease in hydrogen ion concentration." They further state that the increase in titratable acidity of the milk was due to the hydrolysis of the protein, and the consequent increase in the buffer action against added alkali. Clark (9), 1915, defines a buffer as "any substance which tends to preserve the original hydrogen ion concentration of its solution upon the addition of an acid or a base".

Volatile Acid Content.

The amount of volatile acid formed by Bacillus cereus in milk in eight days varied from a "reaction" of 0.2 to 1.4. In twenty days the "reaction" or cubic centimeters of N/10 alkali required to neutralize 100 cc of the medium, was 3.4. No increase in any individual volatile acid could be demonstrated in the distillate from the culture, with the exception of

of the medium unacidified. Indole formation was therefore indicated for the neutral distillate of sterile milk did not give the indole test.

No hydrogen sulphide or mercaptan were formed in twenty days.

TABLE IV
BACILLUS CEREUS*

	Days of Growth						
			8			20	
	A	B	C	D	E	F	G
†Nitrogen as ammonia	3.92+	4.2+	3.92+	6.72+	4.2+	12.32+	12.30+
†Amino nitrogen Denis & Minot	38.0+	23.67+		25.74+	29.08+	57.78+	60.83+
†Amino nitrogen Harris		24.76+	36.69+	30.36+			85.27+
†Reducing substances		0.11+	0.27+	0.72+	0.11+	0.11-	0.7-
p^H				0.3-		1.2-	1.0-
§Total acid	3.2+	2.4+	2.0+	2.2+	3.4+	2.0+	7.0+
§Volatile acid	1.0+	1.4+	1.3+	0.2+	1.0+		3.4+
Age of seed culture in days			48	11	19		2
No. of samples of milk	V	VI	XI	XIX	XX	II	XXIV

* The sign + indicates an increase in the content of the medium.
The - sign indicates a decrease in the content of the medium.
† Milligram per 100 cc of the medium
‡ Calculated as percentage of lactose.
§ Cubic centimeters of N/10 KOH required to neutralize 100 cc. of the medium

(2) Bacillus albolactus.

In Table V the changes produced in milk by the action of Bacillus albolactus are shown. The three analyses were made at different times.

Ammonia and amino nitrogen.

Nitrogen as ammonia formation was constant, but not progressively so. In eight days the rise was 3.36 mgms. and 2.8 mgms. per 100 cc of medium, while in twenty days the increase was 1.4 mgms. per 100 cc of medium.

Amino nitrogen showed a progressive increase. In eight days the amounts formed were 25.71 mgms. and 21.18 mgms. per 100 cc of medium, and in twenty days 31.80 mgms. per 100 cc of medium (Denis and Minot). The amount indicated after twenty days by the Wolf and Harris method was 33.55 mgm. per 100 cc of the medium.

Reducing substances.

Reducing substances calculated as lactose fluctuated from a decrease of 0.9 percent to an increase of .47 percent in eight days, and in twenty days showed a decrease of 2.07 percent.

Total acidity.

Hydrogen ion concentration increased in eight days 1.0 and 1.1, and in twenty days 1.1. Total acidity, as shown by titration, increased from 3.6cc to 5.6 cc N/10 in eight days and in twenty days, there was an increase of 5.0 cc N/10 acid per 100 cc of the medium.

Volatile acids.

Volatile acid formation in eight days varied from 1.0 cc to 1.4 cc. N/10 acid per 100 cc of the medium. In twenty days an increase of 2.9 cc per 100 cc N/10 acid of the medium was shown.

The presence of acetic and butyric acid was recognized in the distillate, but the intensity of the color reactions did not distinguish the volatile acid content of the culture from that of the original medium.

Indole, Hydrogen Sulphide and Mercaptan.

No indole, hydrogen sulphide or mercaptan formation through the action of Bacillus albolactus could be demonstrated.

TABLE V

BACILLUS ALBOLACTUS*

	Days of growth		
	8		20
	A	B	C
†Nitrogen as ammonia	3.36+	2.80+	1.4+
†Amino nitrogen Denis & Minot	25.71+	21.18+	31.60+
†Amino nitrogen Harris			33.55+
‡Reducing substances	0.9-	0.47+	2.07-
p^H	1.0+	1.1+	1.1+
§Total acids	5.6+	3.6+	5.0+
§Volatile acid	1.0+	1.4+	2.9+
Age of seed culture in days		26	
No. of sample of milk	IX	XIX	X

*The sign + indicates an increase in the content of the medium.
The sign - indicates a decrease in the content of the medium.
†Milligram per 100 cc of the medium
‡Caloulated as percentage of lactose.
§Cubic centimeters of N/10 KOH required to neutralize 100 cc of the medium.

(3) Bacillus megatherium.

The decomposition products formed by Bacillus megatherium are shown in Table VI. The seven analyses were made at different times.

Ammonia and Amino nitrogen content.

The formation of nitrogen as ammonia was constant and increased progressively in twenty days. In eight days the amounts formed varied from 1.12 mgms. to 2.80 mgms. per 100 cc. In twenty days the amounts formed varied from 4.48 mgms. to 5.52 mgms. per 100 cc of the medium.

Amino nitrogen formation was constant and increased progressively. The amount formed in eight days varied from 19.48 mgms. to 26.51 mgms. per 100 cc of medium, and in twenty days the increase varied from 41.33 mgms. to 70.20 mgms. (Denis and Minot method), and from 46.92 mgms. to 73.64 mgms. per 100 cc of medium (Wolf and Harris method.)

Reducing substances.

There was no increase in reducing substances in eight days, but a constant increase in twenty days varying in amount from 0.21 percent to 1.0 percent.

Total acidity.

Hydrogen ion concentration showed a decrease of 0.2 in eight days and of 0.1 in twenty days. The "reaction" (cubic centimeters of N/10 potassium hydroxide per 100 cc of medium) varied from 0.6 to 2.0 in eight days, and from 2.6 cc to 5.0 cc in twenty days.

Volatile acids.

There was no increase of volatile acids in eight days, but in twenty days, there was an increase varying in amount from 1.5 cc to 2.4 cc. The presence of acetic and butyric acid was recognized in the distillate, but the intensity of the color reactions was not perceptibly greater than in sterile milk.

Hydrogen Sulphide, Indol and Mercaptan Formation.

No indol, hydrogen sulphide or mercaptan formation was observed.

TABLE VI
BACILLUS MEGATHERIUM

Days of growth	8			20			
	A	B	C	D	E	F	G
†Nitrogen as ammonia	1.12+	2.80+	1.96+	1.12+	5.48+	4.48+	5.52+
†Amino nitrogen Denis & Minot		26.51+	24.20+	19.48+	41.33+	44.38+	70.20+
†Amino nitrogen Harris				21.86+		46.92+	73.64+
‡Reducing substances	0	0	0	0	0.21+	0.8+	1.0+
pH		0.2-	0.2-			0.1-	0.1-
§Total acid	0.6+	0.8+	2.0+	2.0+	2.6+	4.0+	5.0+
§Volatile acid	0	0	0	0	1.5+	2.4+	2.0+
Age of seed culture in days		48	3	8			15
No. of sample of Milk	VII	XII	XV	XVI	III	IV	XIX

*The sign + indicates an increase in the content of the medium.
The - sign indicates a decrease in the content of the medium.
†Milligram per 100 cc. of the medium
‡Calculated as percentage of lactose.
§Cubic centimeters of N/10 KOH required to neutralize 100 cc of the medium.

(4) Bacillus mesentericus vulgatus.

In Table VII the changes produced in milk by Bacillus mesentericus vulgatus are given. All of the analyses were made at different dates.

Ammonia and Amino nitrogen formation.

Ammonia formation was progressively constant, ranging from .56 mgms. to 1.68 mgm. per 100 cc of medium in eight days, and from 9.24 mgm. to 10.08 mgm. per 100 cc of the medium in twenty days. Amino nitrogen formation was also progressively constant. In eight days the amounts formed varied from 22.57 mgm. to 44.4 mgm. per 100 cc of the medium (Denis and Minot method), and from 22.17 mgms. to 46.38 mgms. per 100 cc of the medium. (Wolf and Harris method.) In twenty days the amounts of amino nitrogen formed were 93.76 mgm. per 100 cc and 97.36 mgm. per 100 cc of the medium (Denis and Minot method) while the increases as shown by Wolf and Harris' method were 126.87 mgms. and 128.57 mgms. per 100 cc of the medium. Some factor evidently operated in the Wolf and Harris method, which did not enter into the other methods used for determining amino nitrogen. There was possibly a hydrolysis of some of the protein of the culture during the removal of ammonia.

Reducing substances.

There was an increase of reducing substances in eight days varying from .06 percent to 1.16 percent. In twenty days the amounts formed were 1.35 percent and 1.37 percent.

Total Acidity.

Hydrogen ion concentration decreased in all cultures, the amount of decrease varying in eight days from 0.2 to 0.5, and in twenty days being 1.1. "Reaction" increased throughout. The amount formed varied

from 2.6 cc to 3.6 cc. N/10 acid, in eight days, and in twenty days the amounts formed were 4.6 cc and 4.8 cc N/10 acid, per 100 cc of the medium.

Volatile acids.

Volatile acid formation increased both in eight days and in twenty days. The amounts formed in eight days ranged from 0.8 cc to 1.6 cc N/10 acid, and in twenty days ranged from 1.0 cc to 1.4 cc. N/10 acid per 100 cc of medium. Traces of acetic acid were found in the distillate.

Hydrogen Sulphide, Indole and Mercaptan.

The cultures did not have the odor of hydrogen sulphide, but on distillation of eight day cultures, large amounts of lead blackening sulphur were given off in the distillate, which had a strong odor of hydrogen sulphide. Twenty-day cultures showed no trace of this formation of a volatile sulphide which would distinguish the culture from sterile milk.

No indole was formed in twenty days.

No mercaptan was formed in twenty days.

TABLE VII

BACILLUS MESENTERICUS VULGATUS*

	Days of growth.				
	8			20	
	A	B	C	D	E
†Nitrogen as ammonia	0.55+	1.12+	1.68+	9.24+	10.08+
‡Amino nitrogen Denis & Minot	22.57+	44.40+	41.38+	97.36+	93.76+
†Amino nitrogen Harris	22.17+	44.8+	46.38+	128.57+	126.87+
‡Reducing substances	.06+	.23+	1.16+	1.35+	1.37+
p^H	0.4-	0.8-	0.5 -	1.1 -	1.1 -
§Total acid	2.6 +	3.6+	2.6+	4.8+	4.6+
§Volatile acid	0.8 +	1.6+	1.0+	1.0+	1.4+
Age of seed culture in days	6	4	28	4	4
No. of sample of Milk	XXI	XXI	XXI	XXIV	XXIV

*The sign + indicates an increase in the content of the medium
The - sign indicates a decrease in the content of the medium.

†Milligram per 100 cc of the medium

‡Calculated as percentage of lactose.

§Cubic centimeters of N/10 KOH required to neutralize 100 cc of the medium.

(5) Bacillus mesentericus fuscus.

The changes produced in the composition of milk by the action of Bacillus mesentericus fuscus are shown in Table VIII.

The three analyses of eight day cultures were made at different times, but the twenty day cultures were incubated and analyzed on the same dates.

Ammonia and Amino nitrogen.

Nitrogen as ammonia increased progressively. The eight day cultures showed an increase of 1.40, 4.08, and 1.68 mgms. per 100 cc of the medium, and the twenty day cultures showed an increase of 8.40 mgms. per 100 cc. of the medium.

Amino nitrogen formation in the eight day cultures was 19.34 mgm. and 14.21 mgms. per 100 cc of the medium (Denis and Minot) and 20.92 mgms. and 14.60 mgms. per 100 cc of the medium (Wolf and Harris). In twenty days 26.23 mgms. and 20.76 mgms. more formed (Denis and Minot) and 81.77 mgms. and 74.75 mgms. per 100 cc of the medium (Wolf and Harris).

Reducing substances.

In eight day cultures increases and decreases in reducing substances were observed. In two analyses a decrease of 0.35 and 0.38 percent respectively was found, and in a third analysis an increase of 0.27 percent was observed. In twenty day cultures the percentage of reducing substances calculated as lactose showed no change from that of the original medium.

Total acidity.

The amounts of total acid formed in eight days, as shown by titration with N/10 potassium hydroxide, were in two instances 0.6 cc per 100 cc of the medium. In a third eight day culture, a decrease of 0.5 cc per 100 cc.

of medium was noted. In twenty days 3.2 cc and 2.8 cc per 100 cc of the medium were formed.

Hydrogen ion concentration showed a decrease of 0.2, 0.1, and 0.2 in eight days, but in twenty days an increase of 0.2 was obtained.

Volatile acids.

Volatile acid formation in eight days fluctuated. In one culture no change was observed in the medium, while another culture showed a gain of 1.0 cc N/10 acid per 100 cc of the medium, and a third culture a decrease of 0.3 cc N/10 acid per 100 cc of medium. In twenty days there was an increase of 0.2 cc N/10 acid per 100 cc of the medium.

Acetic acid and butyric acid were demonstrated in the distillate, but the intensity of the color reactions appeared no greater than in sterile milk.

Indole, Hydrogen sulphide, and Mercaptan.

No formation of indole, hydrogen sulphide or mercaptan was caused by the action of Bacillus mesentericus fuscus.

TABLE VIII

BACILLUS MESENTERICUS FUSCUS*

Days of growth.

	8			20	
	A	B	C	D	E
†Nitrogen as ammonia	1.40+	4.08+	1.68+	8.40+	8.40+
†Amino nitrogen Denis & Minot		19.34+	14.21+	26.23+	20.76+
†Amino nitrogen Harris.	20.92+		14.60+	81.77+	74.75+
‡Reducing substances	0.35-	0.27+	0.38-	0	0
p^H	0.2-	0.1 -	0.2-	0.2+	0.2+
§Total acid	0.6+	0.5+	0.6+	3.2+	2.8+
§Volatile acid	1.0+	0.3+			0.2+
Age of seed culture in days	39	4	46	12	12
No. of sample of milk	XIII	XVIII	XXII	XXV	XXV

*The sign + indicates an increase in the content of the medium.
The - sign indicates a decrease in the content of the medium.
†Milligram per 100 cc of the medium
‡Calculated as percentage of lactose.
§Cubic centimeters of N/10 KOH required to neutralize 100 cc of the medium.

(6) Bacillus simplex.

The changes in the composition of milk produced by the action of Bacillus simplex are shown in Table IX. The four analyses were made at different times.

Ammonia and Amino nitrogen.

Nitrogen as ammonia formation increased progressively. The amounts formed in eight days were .84 mgms. 1.12 mgms. and 1.4 mgms. per 100 cc of the medium. In twenty days 7.28 mgms per 100 cc of the medium were formed.

Amino nitrogen.

Amino nitrogen increase was progressive. In eight days increases were obtained of 0.67 mgms. 2.09 mgms. and 3.07 mgms. per 100 cc. of the medium, and an increase of 23.48 mgms. per 100 cc. of the medium was found in twenty days (Denis and Minot). By the method of Wolf and Harris, the increase in amino nitrogen in eight days varied from .62 mgms. to 3.05 mgms. per 100 cc. of the medium, while the increase in twenty days was 54.95 mgms. of amino nitrogen per 100 cc. of the medium.

As in the case of Bacillus cereus, Bacillus mesentericus vulgatus and Bacillus mesentericus fuscus, the amount of amino nitrogen formed in twenty days was much larger when determined by the Wolf and Harris method than when determined according to the method of Denis and Minot.

Reducing substances.

Reducing substances calculated as lactose were decreased in two eight day cultures but increased in a third. In twenty days a decrease was noted.

Total acidity.

In eight day cultures a decrease of 0.2 cc, 0.6 cc. and 0.2 cc. N/10 acid per 100 cc of the medium was found, but in twenty days an increase of 1.6 cc N/10 acid per 100 cc of the medium was obtained in "reaction".

Hydrogen ion concentration decreased in eight days from 0.2 to 0.3 and in twenty days decreased 1.2.

Volatile acid.

Volatile acid content of eight day cultures varied from no change from the original medium to 0.6 N/10 acid cc. per 100 cc of the medium. In twenty days 0.8 cc N/10 acid per 100 cc of medium were formed.

Acetic acid was present in the distillate but not in sufficient amounts to distinguish the culture from the original medium.

Indole, Hydrogen sulphide, and Mercaptan.

No indole, hydrogen sulphide or mercaptan formation could be demonstrated by the action of Bacillus simplex.

TABLE IX
BACILLUS SIMPLEX*

	Days of growth 8			20
	A	B	C	D
†Nitrogen as ammonia	0.84+	1.12+	1.4+	7.28+
†Amino nitrogen Denis & Minot	.67+	2.09+	3.07+	23.48+
†Amino nitrogen Harris	.62+		3.05+	54.95+
‡Reducing substances	.07-	0.5-	0.17+	.04-
p^H	0.2 -	0.2-	0.3-	1.2-
§Total acid	0.2-	0.6-	0.2-	1.6+
§Volatile acid	0	0.6-	0	0.8+
Age of seed culture in days	8	17	7	15
No. of sample of Milk	XIV	XVII	XIX	XXV

* The sign + indicates an increase in the content of the medium.
The - sign indicates a decrease in the content of the medium.
†Milligram per 100 cc of the medium
‡Calculated as percentage of lactose.
§Cubic centimeters of N/10 KOH required to neutralize 100 cc of the medium.

(7) Bacillus subtilis viscosus.

The changes in the composition of milk produced by the action of Bacillus subtilis viscosus are shown in Table X. The four analyses were made at different times.

Ammonia and Amino nitrogen.

Nitrogen as ammonia formation varied in eight day cultures from 0 to 3.36 mgms. per 100 cc of the medium. In twenty days nitrogen as ammonia formation was found to be 9.52 mgms. and 10.64 mgms. per 100 cc. of the medium.

Amino nitrogen increased progressively. In eight days 54.27 mgms. and 57.23 mgms. per 100 cc. of the medium were formed (Denis and Minot). In eight days 56.22 and 57.81 mgms. were formed according to the method of Wolf and Harris. In twenty days 92.03 mgms. and 97.98 mgms. per 100 cc. of the medium (Denis and Minot) were contrasted with 105.91 mgms. and 109.13 mgms. per 100 cc. of the medium (Wolf and Harris)

Reducing substances.

Reducing substances calculated as lactose showed a decrease of 0.99 percent, and 0.36 percent in eight days and a decrease of 0.54 percent and 0.92 percent in twenty days.

Total acidity.

The amounts of total acid formed in eight days, as shown by titration with phenolphthalein, were 4.0 cc. and 2.6 cc. N/10 acid per 100 cc. of the medium. In twenty days an increase of 6.2 cc and 5.0cc.N/10 acid per 100 cc. of the medium was obtained.

Hydrogen ion concentration showed a decrease of 0.4 in eight days, and of 0.7 and 0.6 in twenty days.

Volatile acid.

Volatile acid formation in eight days was 0.2 cc. N/10 acid per 100 cc. of the medium. In twenty days 1.2 cc. and 2.8cc. N/10 acid per 100 cc. of the medium were formed.

Butyric, acetic and propionic acids were identified in the distillate, but the intensity of the color reactions did not distinguish the acid content of the culture from that of the original medium.

Indole, Hydrogen sulphide and Mercaptan.

No indole, hydrogen sulphide or mercaptan formation could be demonstrated.

TABLE X

BACILLUS SUBTILIS VISCOSUS*

	Days of growth			
	8		20	
	A	B	C	D
Nitrogen as ammonia	0	3.36+	9.52+	10.64+
Amino nitrogen Denis & Minot	54.27+	57.23+	92.03+	97.98+
Amino nitrogen Harris	56.22+	57.81+	105.91+	109.13+
Reducing substances	0.99-	0.36-	0.54-	0.92-
p^H	0.4-	0.4-	0.7-	0.6-
Total acid	4.0+	2.6+	6.2+	5.0+
Volatile acid	0.2+	0	1.2+	2.8+
Age of seed culture in days	49	6	49	
No. of sample of Milk	XXII	XXVII	XXII	XXIII

*The sign + indicates an increase in the content of the medium. The - sign indicates a decrease in the content of the medium. Milligram per 100 cc of the medium

Calculated as percentage of lactose

Cubic centimeters of N/10 KOH required to neutralize 100 cc of the medium.

(8) Bacillus brevis.

The changes produced in the composition of milk through the action of Bacillus brevis are given in Table XI. The two eight day cultures were incubated and analyzed at the same time as were also the two twenty day cultures.

Ammonia and amino nitrogen.

Nitrogen as ammonia increased progressively from eight to twenty days. In eight day cultures the increases were 5.32 mgm. and 5.6 mgms. per 100 cc of the medium. In twenty day cultures the amounts of ammonia formed were 7.84 and 8.68 mgms. per 100 cc of the medium.

Amino nitrogen showed an increase in eight day cultures of 25.54 mgms. and 25.95 mgms. (Denis and Minot) and 28.27 mgms. and 30.49 mgms. per 100 cc of the medium (Wolf and Harris). In twenty days there was an increase of 34.52 mgms. and of 35.49 mgms. per 100 cc. (Denis and Minot), and an increase of 32.21 mgms. and of 31.60 mgms. per 100 cc. of the medium (Wolf and Harris).

Reducing substances.

Reducing substances calculated as lactose showed a decrease of 0.66 percent in eight days. In twenty days there was an increase of 1.0 percent and of 1.22 percent.

Total acid.

In eight day cultures there was an increase of total acid of 3.2 cc N/10 acid per 100 cc of the medium as shown by titration with N/10 potassium hydroxide. In twenty days the amounts of increase were 2.6 cc and 2.4 cc. N/10 acid per 100 cc of the medium.

Hydrogen ion concentration showed a decrease of 0.7 and of 0.6 in

eight days, but showed no change from the original medium in twenty days.

Volatile acid.

The amounts of volatile acid formed in eight days were 2.2 cc N/10 acid per 100 cc of the medium, and 7.4 cc. N/10 acid per 100 cc. of medium in twenty days. Acetic acid was present in the distillate.

Indole, Hydrogen sulphide and Mercaptan.

The formation of indole through the action of Bacillus brevis was marked. No hydrogen sulphide or mercaptan formation could be demonstrated.

TABLE XI
BACILLUS BREVIS

	Days of growth			
	8		20	
	A	B	C	D
†Nitrogen as ammonia	5.32+	5.6+	7.84+	8.68+
†Amino nitrogen Denis & Minot	25.54+	25.95+	34.52+	35.49+
†Amino nitrogen Harris	28.27+	30.49+	32.31+	31.60+
‡Reducing substances	0.66-	0.66-	1.0+	1.22+
pH	0.7-	0.6-	0	0
§Total acid	3.2+	3.2+	2.6+	2.4+
Volatile acid	2.2+	2.2+	7.4+	7.4+
Age of seed culture in days	3	3	67	67
No. of sample of Milk	XXVI	XXVI	XXIII	XXIII

*The sign + indicates an increase in the content of the medium.
The - sign indicates a decrease in the content of the medium.
† Milligram per 100 cc of the medium.
‡ Calculated as percentage of lactose.
§ Cubic centimeters of N/10 KOH required to neutralize 100 cc. of the medium

Summary

During the progress of these investigations it has been shown that certain changes are produced in milk by aerobic spore-bearing bacteria. This decomposition is evidently not confined to one of the constituents of milk but includes both proteins and carbohydrates.

An increase in the ammonia content of milk was produced by all aerobic spore-bearing organisms studied. This increase became progressively larger from the eighth to the twentieth day of growth in all cultures studied with the exception of Bacillus albolactus. In the case of Bacillus albolactus the amount of ammonia formed in twenty days was not so great as that formed in eight days.

The amounts of amino nitrogen were uniformly greater the longer the milk was inculated. The change in amino nitrogen content of the milk is undoubtedly the great and significant one. The relative rate of increase, however, varies with different organisms studied. This may indicate as De Bord has said differences in the type of nitrogenous metabolism.

Only two of the organisms Bacillus cereus and Bacillus brevis carried the decomposition to the production of indole. Bacillus mesentericus vulgatus produced hydrogen sulphide in eight days, but was not observed in twenty day cultures.

The estimation of reducing substances in the inoculated milk did not offer much information as to the process going on, possibly owing to the many complicating factors. If the test organism is capable of fermenting lactose we would suppose that the reducing substances in that sample would decrease, and the acidity would increase. Of the organisms studied only Bacillus albolactus and Bacillus brevis are able to ferment lactose. In the tests on both of these organisms there is after eight days a decrease

in reducing substances, and a fairly high acidity. In the case of Bacillus brevis after twenty days there is an increase in reducing substances, and a decrease in acidity as compared with an eight day culture. Bacillus albolactus shows a decrease in reducing substances in twenty days, and a decrease in total acidity as compared with an eight day culture. This decrease in acidity may be explained by the decrease in free ammonia which was also noted in the twenty day culture.

The hydrogen ion concentration may not parallel the changes in titratable acidity as in the case of Bacillus cereus. This apparent inconsistency is discussed on page 49.

Those who have studied putrefaction, particularly Rettger, have maintained that true putrefaction, the production of a foul odor, is brought about only by anaerobic organisms. It is shown in this study, however, that there is close similarity between the action of aerobic and anaerobic spore-forming bacteria on milk since both decompose the protein to amino nitrogen, ammonia, indole and hydrogen sulphide. The one point of difference is that aerobic spore-bearing organisms consistently fail to form mercaptans.

Conclusions

1. The aerobic spore-bearing bacilli investigated showed a constant and progressive increase in ammonia formation.
2. A larger, and also constant and progressive increase in amino-nitrogen formation accompanied the ammonia formation.
3. The actual amount of amino nitrogen present in a solution varied with the method of preparing the solution.
4. Decomposition of the proteins of milk by some of the aerobic bacteria studied proceeded beyond amino acids to indole formation.
5. Hydrogen sulphide formation was also noted in some cases.
6. Mercaptan formation was not observed in the cultures investigated.
7. A decrease in hydrogen ion concentration accompanied an increase in titratable acidity in all cultures of aerobic spore-bearing bacteria studied with the exception of Bacillus albolactus.
8. The action on milk of the aerobic spore-bearing bacilli is in many respects similar to that of the anaerobic bacilli.

BIBLIOGRAPHY

1. Agulhon, H. 1913 Solubilite' de certains sels metalliques des acides gras volatils dans les solvants organiques,- Applications á la détermination qualitative de ces acides. Bull. Soc. chim. series 4, 13: 404

2. Berman, N. and Rettger, L. F. 1918 Bacterial nutrition: Further studies on the utilization of protein and non-protein nitrogen. Jour. Bact. 3: 367

3. Berg 1904 Composition of Milk. Thesis Columbia University

4. Bienstock 1899 Untersuchungen über die Aetiologie der Eiweissfäulniss. Arch. f. Hyg. 36:335

   Bienstock 1901 Untersuchungen über die Aetiologie der Eiweissfäulniss II Milchfäulniss,Verhinderung der Fäulniss durch Milch. Darmfäulniss. Arch. f. Hyg.39:390

5. Bock, J. C. 1916-17 The estimation of amino acid nitrogen in blood Jour. Biol. Chem. 28:357

6. Boussingault 1850 Annales de chimie et de physique 29:472

7. Bushnell, L. D. 1922 Quantitative determination of some of the biochemical changes produced by a saprophytic anaerobe Jour. Bact. 7: 373

8. Cannon, P.R. 1916 A rapid and simple indol test. Jour. Bact. 1: 535

9. Clark, Wm. 1915 The "reaction" of bacteriologic culture media Jour. Inf. Dis. 17: 109

10. Clark, Wm. 1921 The determination of H-ions

11. Clark, Wm. and Lubs, H.A. 1917 Improved chemical methods for differentiating bacteria of the coli-aerogenes family. Jour. Biol. Chem. 30: 209.

12. Clark, Wm. and Lubs, H.A. 1917 The colorimetric determination of hydrogen ion concentration, and its applications in bacteriology. Jour. Bact. 2: 1, 109,191.

13. De Bord, G.G. 1923 Certain phases of nitrogenous metabolism in bacterial cultures. Jour. Bact. 8: 7

14. Denis, W. and Minot, A.S. 1919. Methods for the quantitative determination of the non protein nitrogenous constituents of milk. Jour.Biol.Chem. 37: 353.

15. Descour, L. Pasteur and his work

16. Ducleaux 1900 Dosage des principaux products de la fermentation alcoolique. Traité de Microbiologie, 3: 394.

17. Dyer, D. C. 1916 A new method of steam distillation for the determination of the volatile fatty acids, including a series of colorimetric qualitative reactions for their identification. Jour. Biol. Chem. 28: 445

18. Faulk, G.K., Baumann, E.M. and McGuire, G. 1919 The chemical investigation of spoiled meat Jour.Biol. Chem. 37: 525

19. Flügge, C. 1894 Die Aufgagen und Leistungen der Milchsterlisirung gegenüber den Darmkrankheiten der Säuglinde. Zeitschr. f. Hyg. 17, 272.

20. Folin, O. 1901
Zeitschr. f. physiol. Chem. 32: 515

21. Folin, O. 1902-03 Eine neue Methode zur Bestimmung des Ammoniaks im Harne und anderen thierischen Flüssigkeiten. Zeitschr. f. physiol. Chem. 37: 161

22. Folin, O. 1922 A new colorimetric method for the determination of amino acid nitrogen in blood. Jour. Biol. Chem. 51:377

23. Folin, O. and Bell, R.D. Applications of a new reagent for the separation of ammonia.
Jour. Biol. Chem. 29: 329

24. Folin, O. and Denis, W. 1912 New methods for the determination of total non protein nitrogen, urea and ammonia in blood. Jour. Biol. Chem. 11: 527

25. Folin, O. and Denis, W. 1918 The determination of lactose in milk Jour. Biol. Chem. 33:

26. Folin, O. and Macallum, A.B. 1912 On the determination of ammonia in urine. Jour. Biol. Chem. 11: 523

27. Ford, W.W. and Pryor, J.C. 1914 Observations upon the bacteria found in milk heated to various temperatures. Johns Hopkins Hosp. Bull. 25: 270.

28. Ford, W.W. and Williams, G.H. 1919 Observations on the production of an anti-haemotoxin for the haemotoxin of Bacillus Welchii (Bacillus aerogenes capsulatus) Jour. of Immun. 4: 385

29. Foster, C.F. 1921 The relation of hydrogen ion concentration to the growth, viability, and fermentative activity of Streptococcus hemolyticus Jour. Bact. 6: 161

30. Greenwald, I. 1915 The estimation of non protein nitrogen in blood. Jour. Biol. Chem., 21: 61

31. Harris, J.E.G. 1919-20 The bio chemical comparison of micro organisms by quantitative methods. Jour.Path. and Bact. 23: 30

32. Hawk, P. B. 1917 Practical Physiological Chem.

33. Hertes, C. and Foster, L.M. 1906-07 On the separation of indole from skatole, and their quantitative determination. Jour. Biol. Chem. 1: 257

34. Jordan, E.O. 1921 General Bacteriology

35. Kendall, A.J. 1916 Bacteriology, general, pathological and intestinal.

36. Kendall, A.J. 1922 The significance and quantitative measurement of the nitrogenous metabolism of bacteria. Jour. Inf. Dis. 30: 211

37. Kendall, A.J. and Farmer, C. 1912 Studies in bacterial metabolism. I. II. III. V. VI. Jour.Biol. Chem., 12:13,19,215, 465, 469.

38. Kendall, A.J. and Farmer, C. 1912-13. Studies in bacterial metabolism VII Jour. Biol. Chem. 13: 63

39. Kendall, A.J., Farmer, C., Bagg, E.P., and Day, A.A. 1912. Studies in bacterial metabolism IV. Jour.Biol.Chem. 12: 219

40. Kendall, A.J., Day,A.A. and Walker, A.W., 1913-14 Observations on the relative constancy of ammonia production by certain bacteria. Jour.Inf. Dis.13:425

41. Kendall, A.J., Day, A.A. and Walker, A.W. 1922 Studies in bacterial metabolism. Jour.Inf.Dis.30:

42. Lawrence, J.S. and Ford, W.W. 1916 Spore-bearing bacteria in milk Jour. Bact. 1: 277

43. Matthews, A.P. 1916 Physiological Chemistry

44. Metchnikoff, E. 1908 Études sur la flore intestinale. Ann. de l'Inst. Pasteur, 22: 59

45. Meigs, E.B. and Marsh, H.L. 1913. Composition of milk. Jour. Biol. Chem. 16: 147

46. Neubauer 1855. Jour. für praktische Chemie: 44: 177

47. Osborne, T.B. and Guest, H. 1911. Hydrolysis of casein. Jour.Biol.Chem. 9: 33

48. Osborne, T.B. and Mendel, L.B. 1915 The comparative nutritive value of certain proteins in growth, and the problem of the protein minimum. Jour.Biol.Chem. 20: 357

49. Plimmer, R.H.A. 1918 Practical Organic and Bio Chemistry

50. Raistrick, H. and Clark, A.B. 1921.
A quantitative study of the aerobic decomposition of tryptophane and tyrosine by bacteria. Bio Chem.Jour.15:76

51. Redfield 1912 A study of hydrogen sulphide production by bacteria. Thesis Cornell University.

52. Rettger, L.F. 1901 The liberation of volatile sulphide from milk on heating. Jour. Am. Physiol. 6: 450

53. Rettger, L. F. 1902 An experimental study of the chemical products of Bacillus coli communis, and Bacillus lactis aerogenes Jour. Am. Physiology 8: 284.

54. Rettger, L.F. 1906 Studies on putrefaction Methods of analysis. Jour. Biol. Chem. 2:

55. Rettger, L.F. 1908 Further studies on putrefaction. Jour.Biol. Chem. 4:

56. Rettger, L.F. and Newell,C.R. 1912-13
Putrefaction with special reference to the Proteus group Jour.Biol.Chem. 13: 341

57. Rice, F.E. Studies on the action of erepsin. Jour.Am.Chem.Soc.37:1319.

58. Richmond 1914 Dairy Chemistry

59. Ritthausen, H. 1872 Jour. prakt. Chem. 5: 215

60. Rivas, D. 1912 Studies on indole Centralbl.Bakt. Abt. I Orig.63:547.

61. Robinson, R.H. and Tartar, H.V. 1917
The decomposition of protein substances through the action of bacteria. Jour.Biol. Chem. 30: 135.

62. Schlösing 1851 Ann. de chimie et de physique 31: 153.

63. Shaffer, P.J. 1903 On the quantitative determination of ammonia in urine. Jour. Am. Physiol. 8: 330

64. Sherman, H.C. 1905 Methods of organic analysis.

65. Sörensen, S.P.L. 1907-08 Enzymstudien. Biochem. Zeitschr. 7: 543

66. Sturges, W.S. and Rettger, L.F. 1922. Bacterial antolysis. Jour. Bact. 7: 551.

67. Stutzer, A. 1851. Jour. Landw. 29: 473

68. Tissier, H. and Martelly 1902 Recherches sur la putréfaction de la viande de boucherie. Ann. de l'Inst. Pasteur 16:865

69. Tissier, H. and Gasching, P. 1903. Recherches sur la fermentation du lait. Ann. de l'Inst. Pasteur. 17: 540

70. Van Slyke, D.D. 1912 The quantitative determination of aliphatic amino groups. Jour. Biol. Chem. 12: 275

71. Van Slyke, L.L. 1917 Proteins of Milk . Allen's Commercial Organic Analysis. 113

72. Vernon, H.M. 1903-04 The peptone splitting ferments of the pancreas and intestine. Jour. Physiol. 30: 330.

73. Vulté, H.T. 1915 Household Chemistry 230

74. Waksman, S.A. 1918 Studies on proteolytic activities of soil microorganisms with special reference to fungi Jour. Bact. 3: 475.

75. Waksman, S. A. 1920 Studies on the metabolism of actinomycetes Jour. Bact. 5:1

76. Wolf, C.G.L. 1918-19 (a) The bio chemistry of Bacillus proteus. Jour. Path. and Bacteriology 22: 289

77. Wolf, C.G.L. 1918-19 (b) The bio chemistry of Vibrion septique. Jour. Path. and Bact. 22: 115.

78. Wolf, C.G.L. 1918-19 (c) The proteolytic action of Bacillus sporogenes (Metchnikoff) and Bacillus Welchii Jour. Path. and Bact. 22: 270.

79. Wolf, C.G.L. and Harris, J.E.G. 1916-17 The biochemistry of Bacillus Welchii and Bacillus sporogenes (Metchnikoff) Jour. Path. and Bact.21:386

80. Wolf, C.G.L. and Harris, J.E.G. 1918-19 The bio chemistry of Bacillus histolyticus Jour. Path. and Bact. 22:1

81. Wolf, C.G.L. and Telfer, S.V. 1917 The acid production of Bacillus Welchii and Bacillus sporogenes Bio Chem. Jour. 11: 197

82. Würster 1877 Centralbl. für Physiologie 1: 485

Henrietta Lisk was born April 4th, 1884 in Grahamville, Fla. She attended Stetson University, Deland, Fla. from 1901 to 1903. In 1903 she entered the Florida State College, Tallahassee, Fla. and received the degree from that institution in 1907 and the M.S. degree in 1909. In 1910-11 she taught in the public schools of Tampa, Fla. In 1911-12 she taught Biology in the high school of Sparta, Tenn., and in 1912-13 she taught Botany and Zoology in the Alabama Girls Technical Institute, Montevallo, Ala. She entered Columbia University in 1914 and received the degree of A.M. in 1917. In 1917-18 she taught Biology and Chemistry in Lambertville, N.J. From 1918-20 she was Professor of Biology and Chemistry in Oxford College, Oxford, O. In 1920 she entered the School of Hygiene and Public Health, Johns Hopkins University, Baltimore, and received the degree of D. Sc. in Hygiene, June 12th, 1923.